Molecular markers in populations of the spiders

Lepthyphantes tenuis and *Enoplognatha ovata*

by

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Abstract

Spiders (Arachnida: Araneae) are important polyphagous predators which limit invertebrate pests in arable crops. To utilise them fully as part of an IPM (integrated pest management) programme a greater understanding of basic population dynamics and ecology is required.

Due to their mobility and size, directly studying the population interactions of many invertebrate species is often impracticable. To infer spider population structure indirectly, this study employed a number of DNA based techniques: RAPD (Random Amplified Polymorphic DNA) analysis; PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) analysis; and the analysis of sequence data. In addition to examining variation *per se*, the sequencing of rDNA (ribosomal DNA) ITS (internal transcribed spacer) fragments allowed the phenomenon of concerted evolution to be studied in spiders for the first time.

Two species commonly found on UK arable land: *Lepthyphantes tenuis* (Blackwall) and *Enoplognatha ovata* (Clerck) were studied. These species exhibit different rates of dispersal; the former a highly dispersive ballooning spider, the latter a more sedentary species, permitting the effect of dispersal on patterns of genetic variation to be examined.

RAPD data were generated via five decamer primers and used to determine levels of variation between and within populations at a local scale (< 100 km). Intra-specific similarity calculated via the Nei and Li similarity coefficient was high (*E.ovata* > 83% and *L.tenuis* > 54%). However, despite this high level of similarity, PCO (Principle Co-Ordinate) analysis of the *E.ovata* similarity data generated a pattern which could be related to the geographical separation of the populations. A less obvious structure was detected by PCO analysis of the *L.tenuis* similarity data. Further statistical analysis revealed that F_{st} (population sub-division) estimates were higher, and heterozygosity (gene diversity) levels lower for the *E.ovata* populations than the *L.tenuis* populations, emphasising that dispersal and gene flow reduce population structure.

Analysis of rDNA was carried out with populations across a wider geographic scale. As an initial screen of variability PCR-RFLP of a rDNA fragment was undertaken, with *L.tenuis* from England, Scotland and New Zealand, and *E.ovata* from Invergowrie, Edinburgh and Elgin. Near identical intra-specific patterns were found. To investigate variation further, multiple ITS1 clones from an individual from each site were sequenced in both species. A total sequence variation of 2.7% was recorded across 10 *E.ovata* clones and 1.5% across 10 *L.tenuis* clones. Sequence variation was not population specific, but consisted of unique mutational events. The low variability across geographically distant populations is indicative of concerted evolution homogenising the rDNA array.

The slightly lower level of variation in *L.tenuis* clones may indicate that dispersal and gene flow act to maintain the homogeneity of rDNA spacers. This was reinforced by sequencing three clones from a single *Bathyphantes gracilis* (Blackwall), a Linyphiid with a less dispersive nature than *L.tenuis*. There was 2.1% variation between the clones, approaching the *E.ovata* figure.

Further analysis of spider rDNA was also undertaken. Firstly, hypothetical secondary structures of the ITS1 regions of *L.tenuis*, *E.ovata* and *B.gracilis* were generated and compared, and areas of similarity which may have a functional basis in terms of rRNA processing identified. Secondly, a phylogenetic analysis was carried out with the ribosomal gene data, and highlighted the need for a thorough assessment of systematic relationships within the Arachnida.

Declaration

I hereby declare that the results presented in this thesis were generated by myself alone and have not been submitted previously for any other degree.

The thesis was composed by myself and work by other authors has been acknowledged appropriately.

Stuart W. A'Hara

Dedication

"Those who educate children well are more to be honoured than parents, for these only gave life, those the art of living well." Aristotle

It would be an honour to dedicate this thesis to my parents, Albert and June, who have not only excelled in the dual roles of parent and teacher, but have also been great friends and companions along Via Vitae. Live long and prosper.

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A/T/C/G	adenine/thymine/cytosine/guanine
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
AP-PCR	arbitrarily primed-PCR
bp	base pair
BSA	bovine serum albumen
C	Centigrade
cf.	compare with (L. *conferre*)
COI/COII	cytochrome oxidase I / cytochrome oxidase II
CO_2	carbon dioxide
DAF	DNA amplified fingerprints
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
Dr	Doctor
Ed(s)	Editor(s)
e.g.	for example
EtBr	ethidium bromide
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbant assay
ETS	external transcribed spacer
HCl	hydrogen chloride
HWE	Hardy-Weinberg equilibrium
i.e.	that is
IGS	intergenic spacer
IPM	integrated pest management
ITS	internal transcribed spacer
kb	kilobase(s)
KCl	potassium chloride
km	kilometre(s)
LB	Luria-Bertani broth
LMP	low melting point
M	Molar
m	metre(s)
MAAP	multiple arbitrary amplicon profiling
$MgCl_2$	magnesium chloride
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar(s)
ms^{-1}	metres per second
mt	mitochondrial
N/A	not applicable
NaCl	sodium chloride
N.B.	note well
NCBI	National Centre for Biotechnology Information

ng	nanogram(s)
nm	nanometres(s)
NTS	non transcribed spacer
PCO	principle co-ordinate
PCR	polymerase chain reaction
pers. comm.	personal communication
pers. obs.	personal observation
pg	picogram(s)
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
Rnase A	ribonuclease A
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SAC	Scottish Agricultural College
SCRI	Scottish Crop Research Institute
SDW	sterile distilled water
spp.	species
s.s.	*sensu stricto* (in the strictest sense)
SSCP	single strand conformation polymorphism
SSRs	simple sequence repeats
STET	sodium chloride/Tris/EDTA/Triton
TAE	Tris/Acetic acid/EDTA
TBE	Tris/Boric acid/EDTA
TE	Tris/EDTA
Tris	Tris (hydroxymethyl) aminomethane
µg	microgram(s)
µl	microlitre(s)
UPGMA	unweighted pair-group method with arithmetic mean
UK	United Kingdom
UV	ultraviolet
V	volt(s)
VNTR	variable number of tandem repeats
w/v	weight for volume

1. INTRODUCTION

1.1. Overview

This introductory chapter has been split into two distinct sections. The first explores the rationale for carrying out a DNA based study on spiders, an often maligned and mis-represented order, and places the aims of the project into a broad ecological context.

The second section reviews a number of methods that may be used to generate population genetic data, then explores the reasons behind the selection of the techniques which were ultimately employed during the study.

1.1.1. Rationale

As a result of steadily growing concern regarding the negative environmental side-effects, in addition to the escalating costs, of employing pesticides to combat agricultural pests, there is increasing pressure towards reducing the level of agrochemical application from both the public, and the farming industry itself (e.g. Lisansky and Coombs 1994; Marsh 1995). Consequently, greater attention is now being paid to increasing the use of natural predators as part of an overall Integrated Pest Management (IPM) approach. The IPM philosophy seeks to find a satisfactory balance between biological and chemical means of pest control (e.g. Hoy and Herzog 1985; Greig-Smith 1992; Kogan 1998).

1.1.2. Spiders as biocontrol agents

For several valid reasons, spiders (Arachnida: Araneae) are considered prime candidates for augmentation and conservation in crops or in adjacent habitats as part of an invertebrate pest suppression strategy (e.g. Reichert and Lockley 1984; Chiverton 1986; Young and Edwards 1990; Topping and Sunderland 1994). They form one of the largest and most ubiquitous group of predators in the animal kingdom - some 34,000 recorded species - with many times that number considered to be currently unclassified (Coddington and Levi 1991), and accommodate representatives in almost every conceivable ecological habitat. Indeed, to underline their tremendous adaptability, one species present in the UK, *Argyroneta aquatica* (Clerck) lives predominantly underwater, using an air-bubble attached to aquatic plants as its residence (Foelix 1982). Pertinently however, in relation to pest control there are many spider species with more "conventional" life-history strategies than *A.aquatica* which are present in abundance in the arable ecosystem, and these will be addressed in detail shortly.

In addition to their ubiquity, spiders occur in relatively high numbers in adult and sub-adult form throughout the year. This can be regarded as an important factor for effective biocontrol, since the constant presence of adult spiders represents an opportunity for suppression immediately pests become active, then continuing on throughout the season. For example, Holland and Thomas (1997) carried out a two year study of predation on cereal aphids in wheat by polyphagous predators and concluded that the activity period of the Araneae (mainly comprised of species of the family Linyphiidae) was the most closely correlated with that of aphid populations.

Notably, in terms of diet spiders feed primarily on insects (although cannibalism, oophagy and kleptophagy do occur (Nyffeler *et al.* 1994)). A lengthy period of crop protection is in contrast to other beneficial invertebrate predators, which may not appear in any significant numbers in crops until pest populations have already reached commercially damaging levels. This has been reported as particularly so for aphid-specific antagonists (Dinter and Poehling 1992).

Spiders can therefore be considered to be acting as a pest buffer, damping the deleterious effects of pests until more specific predators are active, and may actually maintain pest numbers below their potential maximum (e.g. Reichert 1974; Reichert and Lockley 1984; Chiverton 1986). The concept of a buffering capacity emphasises that spiders are not presented as the panacea of pest control. In contrast to more specific biocontrol agents, such as parasitoids, polyphagous predators are thought unlikely to exclude pest species completely as they are unable to closely track large-scale changes in pest densities (due principally to their relatively slower generation time (Reichert and Lockley 1984)). Interestingly though, more recent studies have reported that spiders do appear to congregate and have increased reproductive success in areas of high pest density, possibly indicating some limited ability to react to increased prey numbers (Reichert 1992; Weyman and Jepson 1994). A particular benefit which a number of spider species bring to pest control is their ability to disperse, often in large numbers, into the centre of the crop, and not only be present near the field margins. The dispersal of spiders is discussed further in Section 1.1.4.

The notable discrimination must however be emphasised - spider predation may be important in *determining* the density of insect-pest populations, but not in

regulating density. This is more than a case of semantics. It is because spiders are only rarely found to inflict strong density-dependent mortality upon their prey populations. Indeed, the fraction of pests killed is likely to be independent of their density. However, because the spider complex does kill a substantial fraction of a given population, pest insect populations are more dense in the absence of spiders (Wise 1993; Reichert and Lawrence 1997).

Due to their very nature, the possibility of intraspecific predation occurring amongst generalist predators raises the question of whether or not spiders metaphorically "cut each others' throats", effectively allowing pests to flourish, and this has been reviewed in depth by Wise (1993), and more recently Miyashita (1996). The evidence presented, at least for web-building spiders, suggests that competition should not be of concern in terms of their ability to act as biocontrol agents, not due to niche separation (i.e. differences in web placement) although this does occur to a great degree, but because spiders do not typically reach densities high enough for competition to make food limiting. In support of this, in a study in arable crops, webs were found on almost all occasions to contain uneaten aphids, indicating that food availability is not a constraint. Admittedly, this argument is weakened somewhat by the evidence that large quantities of aphids in the diet may be toxic to some species of spider (Toft 1995), and therefore uneaten aphids may have been present even under conditions of food shortage if the spider was reaching a toxic level. On balance however, this seems unlikely. Furthermore, directly observed incidences of spider cannibalism are reported as rare (Samu *et al.* 1996). However, the notion that spiders do not reach sufficient densities to make prey limiting must not be misconstrued as an

indication that they are uncommon or ineffectual as predators. It must be remembered that spiders generally have a very low rate of metabolism and have the ability to store energy and endure periods of starvation for considerable lengths of time (the foraging strategy of "sit and wait" is a very low energy regime (Anderson 1970)). Therefore, spider density can be high without prey becoming limiting.

Interestingly, a further factor which can be added into the equation of spiders as biocontrol agents is the high level of wasteful pest killing that may occur. That is to say, prey which is not necessarily eaten directly by spiders, but is removed from the pest population by becoming ensnared in abandoned webs, or captured in an occupied web but left uneaten. These features, in relation to the linyphiid spider *Lepthyphantes tenuis* (Blackwall), are cited by Sunderland (1996) as factors which make this spider species particularly conducive to pest control. Furthermore, there is a currently unsubstantiated theory that the mere presence of spiders may have an indirect effect on pest invertebrates, causing them to settle for a shorter time and hence render less damage (Reichert 1997).

As broached at the beginning of this chapter, the theoretical potential for spiders to reduce pests in an agricultural setting has been postulated many times over the past few decades e.g. Nyffeler and Benz (1981) hypothesised that although periodic destruction of the vegetation in cultivated fields limits the potential of web builders as pest control agents, ground wandering spiders reach high densities and may "stabilise certain insect populations of meadows and cereal fields".

Importantly, there are also a large number of practical studies which provide empirical evidence of the beneficial impact of spiders, substantiating the theoretical

claims. Indeed, although spiders are typically present at a lower density in agroecosystems than natural grasslands (e.g. Topping and Lovei 1997), they have nonetheless been found to exert considerable control on agricultural pest populations in a number of different arable ecotypes across the globe.

Examples of the positive effect of spiders include studies at the International Rice Research Institute in the Philippines and South China, which have shown that many rice pests are heavily preyed upon by spiders, and in some experiments, spider-induced mortality rates of a major pest, the Brown Planthopper, reached almost 70 % (Hill 1987). Similarly, Mansour *et al.* (1980) working in Israel, recorded that larval populations of the apple pest *Spodoptera littoralis* did not develop to damaging proportions on trees occupied by spiders, and indeed further work by the same researchers reported that 98 % of larval *Spodoptera* spp. mortality was caused by spiders. Reichert and Bishop (1990) presented clear evidence that by manipulating the habitat in a mixed-vegetable garden (by introducing mulches and flowers between the rows as refuges) spider numbers could be increased, which in turn reduced pest abundance and damage to the vegetable crops. Young and Lockley (1985) observed that the lynx spider, *Oxyopes salticus* (Hentz), played an important role in maintaining several pest species at low levels in agroecosystems - but stressed that this species "cannot control major pest outbreaks" alone.

This is a recurring theme when considering spiders as biocontrol agents - it is almost always the assemblage of spiders in a particular habitat, rather than a single species, which is thought to be important. The "assemblage effect" can be considered to be due to the direct relationship between predator size and prey capture - in effect,

the greater variety in size of predators, the greater size and shapes of prey species which will be consumed (e.g. Nentwig 1982; Provencher and Reichert 1994; Reichert and Lawrence 1997).

Notably, not all studies have wholeheartedly supported the case of spiders as effective biological control agents, since the presence of a pest species in the diet of spiders is not proof *per se* that they are effectively reducing pest populations. Sanderson *et al.* (1992), for example, have challenged the supposition that spiders directly affect aphid numbers. Their field study in the South of England surmised that the weather was 80 % responsible for aphid decline, and that polyphagous predators, including spiders, acted simply to "mop up" an already declining population. However, such studies are few and far between, and the majority of published literature has tended to support the beneficial role of polyphagous predators on the whole, and due to the many aforementioned benefits, spiders in particular (e.g. Wise 1993 and references within; Nyffeler *et al.* 1994). The overwhelming conclusion from studies analysing the effects of spiders on invertebrate pest is a negative correlation between spider and pest density.

Having presented a number of examples from the literature which emphasise the benefit of spiders in a number of diverse environments, attention will now be focused on the linyphiid spiders, and their utility towards cereal crop protection.

1.1.3. Linyphiid spiders

Linyphiid spiders (Araneae: Linyphiidae), are the numerically dominant spiders in arable crops in Europe, and are important in the control of cereal aphids on winter wheat (e.g. Sunderland *et al.* 1986; Alderwieredlt 1994). They can reach densities of several hundred individuals per m^2 at the height of summer (Dinter and Poehling 1992), and despite their small size (< 3 mm) mid-summer web-coverage greater than half the surface area of the field has been recorded (Sunderland *et al.* 1986). The webs take the form of non-sticky horizontal sheet-webs, from which the spider hangs, waiting for vagrant prey. The spider then pulls the prey through the web and uses its chelicerae and venom glands to immobilise its victim. Death does not come quickly! Spider mouthparts are not designed to ingest solid food - they inject digestive enzymes into the immobilised prey and then suck the dissolved tissue in liquid form. Linyphiid spiders do not recycle their webs, and as mentioned previously, the webs can ensnare prey even following abandonment.

As evidence of the efficacy of linyphiids as a pest control agent, Kennedy (1990) employed the ELISA (enzyme linked immunosorbant assay) technique to examine *L.tenuis* in cereal fields in Ireland for the presence of cereal aphids in the gut. Some 92 % of females and 76 % of males collected by pootering were positive (cited in Sunderland 1996). In further support, Alderwiereldt (1994) reports that in arable systems aphids can form 55 % of total prey items encountered by linyphiids and some 37 % of total prey consumed.

Often referred to as money spiders (according to folk-lore because of the shimmering, silvery appearance of their silken thread), linyphiids are divided by many

authors into two groups, given sub-family rank; the Erigoninae and the Linyphiinae. However, in reality, no sharp division can be drawn between the sub-families as there are some genera which fall within the morphological extremes of both. Nevertheless, the distinction is made for convenience and is based primarily on the presence of one or two dorsal spines on the fourth tibia - representing the Erigoninae and Linyphiinae respectively. To identify a species accurately however, a number of other morphological features must be examined (Table 1.1.)

Table 1.1 Main morphological characteristics used for spider identification

Characteristic	Example
Tibial spines	Roberts (1987)
Metatarsal trichobothrium	Roberts (1987)
Male palp structure	Merret (1963), Millidge (1977)
Epigyne structure	Locket and Millidge (1953)
Tracheal system	Blest (1976)

Typically, Erigonines build sheet webs at ground level across depressions in the soil, whereas Linyphiinines build webs above ground level (with adults closer to the soil than juveniles) using vegetation to support their webs (Samu *et al.* 1996). The stratification of webs in the vegetation enables a number of individuals to share the same temporal space, which may increase the efficiency of pest capture (pers. obs.). Interestingly, progeny of the aphid *Rhopalosiphum padi* are almost exclusively found on stem bases during the earliest stages of crop growth and aphid infestation (Chiverton 1986) - this proximity to both adult Linyphiinine and Erigonine web sites may indicate one reason for linyphiid success as an aphid predator.

L.tenuis is the most numerous linyphiid present in the UK (Bristowe 1958) and is particularly common in grass and cereals. The species is active all year round,

breeding continuously between March and November (Topping and Sunderland 1994) and overwinters in the relative protection of leaf litter. Females in the laboratory have been recorded to lay an average of 10 egg sacs per year (C.J. Topping pers. comm.) and these will contain between 10-20 spiderlings in each (pers. obs.). Spiderling mortality in the field is thought to be high however, with both environmental (temperature and moisture extremes) and biological pressures (predation and parasitism), responsible in part (Sunderland 1996).

Linyphiids, like all spiders are dioecious i.e. separate sexes, and are diploid. Sex determination is dependent on the sperm cell, which after meiosis either carries a single set of sex chromosomes, giving rise to a female; or no sex chromosomes, giving rise to a male (the female always contributes a set) (Foelix 1982).

1.1.4. Ballooning in spiders

In addition to being highly fecund, *L.tenuis* is also highly dispersive. It is a common "ballooning" species, dispersing aerially by exuding a silk line when environmental conditions are suitable i.e. wind speed < 2 ms^{-1} (Greenstone 1990; Topping *et al.* 1992) (N.B. The term migration will be deliberately avoided when describing spider dispersal to prevent any notion of intended directionality). Ballooning typically takes place in the morning when the sun heats the ground, producing rising thermals of air. Indeed, it has been hypothesised that it is this change in air temperature which forces the spiders to escape from these unfavourable conditions (or conversely, it may simply be that the thermals create a favourable "launch window"). To initiate this remarkable form of dispersal, a spider will climb to an eminence and adopt a posture anthropomorphically equivalent to standing on tip-

toe, with its abdomen in the air, and begin to release a silk line from its spinnerets. As more silk is released, wind-drag builds up along the length of the line until the force exceeds the spider's body weight, and the individual becomes airborne. Often large numbers of individuals will balloon simultaneously, creating a memorable sight, perhaps described most poetically by the ancient scholar Pliny, who commented on a period of mass ballooning as the day; " ...it rained wool" (cited in Bristowe 1939).

The exact trigger which motivates ballooning is unknown, and recent papers fuel the causal debate. Weyman *et al.* (1995) suggest that ballooning behaviour may be a function of population density on the ground, rather than any seasonal cycle in motivation, but this is in contrast to the work of Topping and Sunderland (1998) which suggests, certainly in the case of female *L.tenuis,* that it is not population density but environmental conditions which control ballooning i.e. if a suitable environmental window appears, ballooning will occur independently of population density. Yet further studies have shown that by limiting food supply ballooning frequency can be greatly increased (e.g. van Wingerden 1978), and in concordance a recent paper by Weyman and Jepson (1994) has shown that spiders arriving in barley artificially supplemented with aphids are more likely to remain in the short-term - particularly immature spiders. In reality, it is probably a number of factors which induces ballooning and further studies are required to settle the issue. Interestingly, whatever mechanism(s) triggers the phenomenon, ballooning in linyphiids is not tied to a specific stage in the life cycle, as is the case with some members of the Insecta e.g. the larval dispersal of the spotted stem-borer *Chilo partellus* (Berger 1992), but occurs in both immature and adult spiders. Notably in terms of crop protection, adult

L.tenuis have been found to be more likely to balloon than immature spiders (Weyman *et al.* 1995). As a consequence of this, adults spiders will balloon into crops and be able to reproduce without any time lag to reach sexual maturity.

The distances spiders achieve via this mode of dispersal is unknown, but it is not inconceivable that large distances can be covered on occasion. There are, for example, several incidences of spiders, both adult and juvenile, collected in trawl samples taken by aeroplanes at many thousands of feet, indicative of great potential for dispersal (Foelix 1982). Perhaps most famously, Charles Darwin, aboard the research ship Beagle, noted that whilst the ship was at least 60 miles from the South American coast, the masts and rigging were strewn with the silken threads of thousands of small ballooning spiders (Darwin 1879).

Indeed, to further this point, in a study on the arrival and primary establishment of spiders following the Mount St. Helens volcanic eruption in the USA, Crawford *et al.* (1995) believe that their data, which recorded high rates of immigration by ballooning species, indicate that aerial dispersal is of prime importance in the recolonisation of devastated terrain, and may well have been underestimated in other situations. This belief was based on the colonisation of the volcanic pumice plain by several ballooning spider species, including an Erigonine, *Walckenaeria pellax* (Millidge), whose nearest realistic source population was over 50 km away.

It may be a less spectacular example than volcanic devastation, but in a nonetheless comparable situation, the ability to balloon into new territory, and accompanying high reproductive rate of linyphiids, potentially make them an

excellent coloniser in the ephemeral agroecosystem, which periodically undergoes dramatic changes due to agricultural practices. Ploughing for example has a particularly devastating effect (> 90 % mortality) on spider populations (Topping and Sunderland 1994), as similarly does the application of pesticides. Ironically, in stark contrast to their beneficial nature, linyphiids in temperate arable crops are amongst the most sensitive indicators of pesticide side-effects, consistently being recorded as the most negatively affected invertebrate group in a survey on the impact of three pesticides (Everts *et al.* 1989). In a further study, mortality rates of > 80 % were recorded amongst linyphiid spiders following spraying of the pesticide deltamethrin (Thomas *et al.* 1990).

The predisposition towards ballooning of adults, coupled with the high reproductive capacity facet of their life-history strategy, may indicate why *L. tenuis* has prospered as more and more acreage has been put under agriculture in the last few centuries. The frequent ballooning behaviour has been hypothesised as a life history strategy which spreads "biological risk" by enabling female *L.tenuis* to lay egg sacs in different localities, giving her offspring a greater chance of survival (Weyman and Jepson 1995; Samu *et al.* 1996). This would be a typical strategy of a species which thrives in constantly disturbed habitat such as the agroecosystem (e.g. Greenstone 1982; MacDonald and Smith 1990). Indeed, a life-history strategy of this nature is similar to that of an agricultural weed and, as is hypothesised for weed species, it may be that *L.tenuis* was once a rare species, adapted to naturally occurring ephemeral patches, and is therefore pre-adapted to survive and flourish in the ever increasing ephemeral agricultural landscape (Topping and Sunderland 1998).

Whatever the aetiology of the ballooning habit, it is one which can deliver spiders, both adult and immature, directly into the heart of the crop, and is therefore of great interest for crop protection.

1.1.5. Modelling land-management practices to promote spider populations

Furnished with data on the phenomenon of spider ballooning, it is reasonable to hypothesise that in an agricultural context the recolonisation of arable crops following agricultural practices will be a function of the distribution of undisturbed sources of immigrant populations in the surrounding landscape. Furthermore, since the literature emphasises that the presence of linyphiid spiders in crops is beneficial in curbing invertebrate pests, particularly aphids, a land-use rationale which supports or augments the population density of spiders, i.e. which increases the potential sources of immigrants, is to be sought. Ideally, to test assumptions before implementing a plan of action, a computer simulation model should be designed which will enable a prediction of how spider populations may react to changes in the timings of agricultural practices, and give an indication of how land-use may affect spider density. Such models will be of benefit, not only in modelling the utilisation of the landscape to maximise spider populations for biocontrol purposes, but also in gaining understanding of more fundamental areas of spider biology, such as population structure and bio-diversity across landscapes (with the latter becoming increasingly topical in terms of devising conservation strategies for endangered species i.e. how local extinction and colonisation rates are effected by patch size and isolation).

Topping and Sunderland (1994) produced a spatial metapopulation dynamics model incorporating available ecological information obtained from experimental results and published literature (for example, data on reproductive rate, maturation rate, adult and juvenile mortality constants and adult and juvenile dispersal constants in a number of different habitats), which predicted the effect of land use on the population density of *L.tenuis*. The classical metapopulation model of population structure (as defined originally by Levins 1970; Hanski and Gilpin 1991) states that field populations are considered to be demes (hypothetical division of reproducing units) within a set of interacting local populations in which all demes are equally prone to extinction, with the overall metapopulation surviving through recolonisation. The main departure of metapopulation models over prior population models was the removal of the assumptions of population immortality and fixed population size.

Due to a lack of quantitative data in their model, ballooning distances were modelled with a simple dispersal function (tantamount to diffusion). Consequently, dispersal from a population during any month was modelled as being equally distributed in all directions, with individuals only travelling to adjacent plots. This assumption is supported by Van Wingerden (1980) and Miller (1984), who assert that aerial dispersal is usually a short-range phenomenon, possibly in response to very local stress factors (although as Crawford *et al.* (1995) demonstrated, long range dispersal is perhaps not uncommon).

At this point it is interesting to note that despite their long recognised potential as pest control agents, relatively few ecological studies on spiders have been

carried out. This may be due in part to the difficulty of studying small, highly mobile invertebrates, and perhaps to a lesser extent in part to the less than appealing press spiders typically receive.

Crawford *et al.* (1995) briefly touch on the possibility of spiders existing in a metapopulation framework due to their findings of large dispersal magnitude and the known habitat specificity of some spiders, and Toft and Schoener (1983), although not explicitly outlining the structure as a metapopulation, uncovered patterns of extinction and colonisation of species of orb spiders on Bahamian islands that could be interpreted as a metapopulation.

Notably, Gilpin (1991) simulated the population dynamics of a hypothetical three patch (i.e. three population) classic metapopulation which resulted in a precipitous drop in genetic heterozygosity. Few metapopulations connected by dispersal exhibit very low levels of genetic heterozygosity in nature, and thus classic metapopulations are considered rare. Perhaps a more realistic, if not particularly helpful, definition of a metapopulation is any set of conspecific populations, possibly - but not necessarily interconnected! Harrison (1991) suggests each case, with each new species, must be evaluated in its own right whilst paying careful attention to alternative hypotheses.

Interestingly, in the case of the Topping and Sunderland (1994) model, metapopulation survival *was* found to be possible even under conditions of considerable disturbance (i.e. despite local extinctions, the population as a whole survived), due to the immigration of spiders from nearby plots. Furthermore, the simulation proceeded to demonstrate that the overall effect of incorporating areas of

set-aside into the arable landscape depends on the management of the set-aside and its positioning within the landscape. It was found that projected population densities of *L.tenuis* were highest in populations within non-rotational set-aside, which in turn would increase density in surrounding arable crops by acting as stable islands for spiders to reproduce in and disperse from. The effect was greatest when the set-aside was randomly distributed in the arable landscape. More recently, Halley *et al.* (1996) have produced a spatial dynamics model taking a similar approach to Topping and Sunderland's (1994) paper, but modelling the effects of different levels of pesticide application on spider abundance and persistence across a number of habitat types, rather than the effects of physical disturbances. They did not include set-aside as a habitat type under consideration, but concluded that heterogeneity in the landscape, and in particular the inclusion of small patches of grassland in areas of intense cereal production, dramatically increased spider numbers. Halley and co-workers (1996) also concluded that rotation reduces average population size. There are fewer studies recording the effects of disturbances in perennial crops on spider populations (by nature perennials undergo fewer disturbances than annual crops), but the application of pesticides would inevitably have a seriously detrimental effect to all arthropods, including spiders. In this case, as with arable crops, the proximity of regions of unaffected spiders would again be important for re-colonisation.

Both these models seem to indicate a "spider friendly" course of action in respect to set-aside and grassland management within an arable landscape, but their outcomes are based on the presupposition of dispersal as diffusion into adjacent plots. The metapopulation model proposed in these simulations may have achieved

population stability, but we do not have data to confirm that this is indeed the situation which is actually occurring in the field (although given the data on ballooning, intuition strongly points in that direction).

The classical metapopulation is considered to be one in which individuals *infrequently* move from one location (population) to another at substantial risk of not finding another suitable habitat patch (Hanski and Gilpin 1991). However, recent work (Topping and Sunderland 1998) indicates that the main factors preventing female *L.tenuis* from ballooning are the environmental conditions, not food availability or any perceived 'quality' of site, and given appropriate conditions females will balloon out of a given area immediately. This points not towards *infrequent* interaction between populations, but to *frequent* dispersal, removing one major tenet of the classical metapopulation scenario. Secondly, metapopulations are thought to exist when habitat specificity is high. If agricultural crops and permanent grassland are defined as potential linyphiid spider habitat, then some 55 % of the UK landscape can be considered suitable (Bunce and Heal 1984) - removing a secondary tenet of the classic metapopulation. There are however, a number of possible strings to the metapopulation bow.

1.1.6. Gene flow and population structure

The ability that ballooning confers to disperse readily across the landscape leads to a number of possible hypotheses regarding the structure of linyphiid populations. It can be proposed that *L.tenuis* populations may not exist in the framework of a classical metapopulation, but may exist as a "global", or panmictic population, in genetic terms, with *no* demarcation of conspecific populations.

However, a true global genetic structure is perhaps unrealistic for all but a few species whose members return to a single point for mating. More plausibly, a situation akin to the "*n*-island" model of population structure, in which every *local* sub-population is equally accessible from every other, may exist (Slatkin 1985). In metapopulation terms this is comparable to a patchy "pseudo-metapopulation" and would lead to reduced genetic structure due to high levels of gene flow (See Figure 1.1).

Other models of gene flow which can be considered include the "continent-island" model, in which there is effectively one-way movement from a large ("continent") population to a smaller, isolated population; the isolation by distance model; and the stepping stone model. The continent-island model can also be interpreted in terms of a metapopulation, since populations may be unequal in size or longevity, creating a more stable mainland gene pool from which individuals can disperse. Indeed, Boorman and Levitt (1973) concluded that this was more likely to occur in nature than the classic metapopulation structure. This is intrinsically how areas of non-rotational set-aside would function in the arable landscape.

The "isolation by distance" model has been the model most often applied to describe population genetic structure. Usually the total population forming a species is not a random mating unit because the distance of individual dispersal is much smaller than the entire distribution range of the species. This phenomenon will lead to local differentiation of gene frequencies due to random genetic drift. Moreover, the tendency for differentiation is considered greater in a linear habitat (e.g. following the bank of a stream or the side of a road) in comparison with a multi-dimensional habitat

(Wright 1943). The "stepping-stone" model of population structure is similar to the framework of the isolation-by-distance model, but in this case each population can receive immigrants only from neighbouring populations. The balance between gene flow and genetic drift will delimit the level of population differentiation. This model can also theoretically reach genetic panmixia if the populations are close to each other and dispersal rates are high. However, these models assume constant population size and population immortality in space.

To re-emphasise, the primary difference between the metapopulation approach, and the continent-island, stepping-stone and isolation by distance models, is that metapopulation models do not assume constant population size or population immortality. This makes the metapopulation approach more relevant to the study of spiders in agroecosystems as patch extinction occurs at regular intervals, as described in Section 1.1.4.

A less likely population structure in terms of linyphiid spiders, outlined by Harrison (1991), is the non-equilibrium metapopulation, which refers to a set of populations in which little or no re-colonisation occurs. This can lead to population differentiation if extinction does not occur. However, this seems the least plausible scenario given the ubiquity and dispersive nature of *L.tenuis*.

Whichever theory is more applicable it may have important implications regarding the modelling of *L.tenuis* population dynamics. Hillis and Moritz (1996) stress that an understanding of population genetic structure must underlie sound species-management decisions. If a panmictic or patchy metapopulation structure is more germane, then the previously described population dynamics model, of

individuals diffusing out only into adjacent patches, will have to be re-considered to take into account the frequent ballooning of females and their relatively unlimited access to other members of the gene pool.

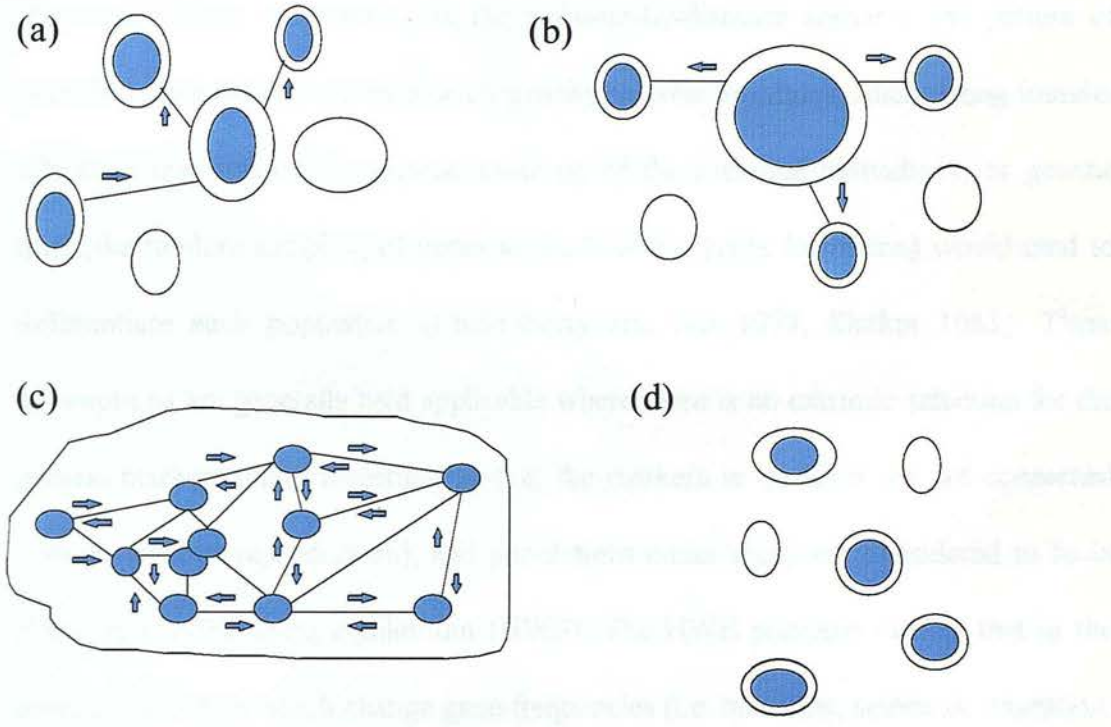


Figure 1.1. Diagram of different metapopulation types. (a) classical; (b) continent-island; (c) patchy population and (d) non-equilibrium. Filled ovals represent occupied habitat patches, unfilled ovals, vacant patches. The black lines represent dispersal and the arrows indicate the direction of dispersal. Outer black ovals encompassing filled ovals represent the boundaries of each local population. From Harrison and Hastings (1996).

1.1.7. Genetic consequences of population interactions

The key to how we may untangle the web of which population model best represents *L.tenuis*, and hence how best we can model population dynamics, lies in the phrase "gene flow". Each of the aforementioned models of population structure may lead to an identifiable population genetic structure, due to the levels of gene

flow within and between populations. By gathering data on the existing population genetic structure of an organism we can then infer information indirectly on past dispersal. For example, a patchy metapopulation structure may result in low levels of genetic structure due to the homogenisation of the gene pool via gene flow (Futuyama 1986). In contrast, in the isolation-by-distance scenario, the pattern of gene flow may result in genetic heterogeneity between localities, since strong founder effects (a bias toward the genetic make-up of the founding individuals) or genetic drift (the random sampling of genes at the time of zygote formation) would tend to differentiate each population (Chakraborty and Nei 1977; Slatkin 1985). These assumptions are generally held applicable where there is no extrinsic selection for the genetic markers under investigation (i.e. the markers in question are not concerned with survival or reproduction), and populations under study are considered to be in allelic Hardy-Weinberg equilibrium (HWE). The HWE principle implies that in the absence of factors which change gene frequencies (i.e. mutation, selection, migration, random drift) and with random mating, the population will immediately arrive at, and remain in, the same allelic proportions. The principle also implies that the population variability remains the same from generation to generation (Crow and Kimura 1970).

The salient question which must be addressed is how to gather information on the current population structure, and hence past gene flow, of these beneficial predators? Linyphiid spiders are too small, and potentially too mobile, to carry out traditional mark, release and re-capture experiments, to directly record gene flow, and so other methods must be considered to study population interactions. Indeed, the direct recording of the movements of individuals does not necessarily produce

information on population interactions, as immigrating individuals may not manage to reproduce within the existing population.

Answers may lie with biochemical and molecular techniques, which provide a powerful set of tools for the study of population interactions by producing data from the most intrinsic set of markers available- the genetic code of proteins and DNA. As can be seen, differences in levels of gene flow (i.e. effective dispersal) can be expected to result in different patterns of genetic variation within and between geographic localities. Therefore the analysis of DNA differentiation (or variation) between populations should to some extent indicate dispersal ability.

1.1.8. *Enoplognatha ovata* - a sedentary spider

In addition to the highly dispersive *L.tenuis*, analysis of the population genetic structure of the relatively sedentary *Enoplognatha ovata sensu stricto* (Clerck), will be carried out as means of a comparison. *E.ovata s.s.* (Araneae: Theridiidae) is a medium sized spider, approximately 5mm in body length, which is distributed widely throughout Europe and coastal North America (Oxford and Reillo 1994). *E.ovata* is a member of a five sibling species group which until relatively recently was thought to belong to a single species polymorphic for sexual organs (Hippa and Oksalam 1982, 1983a). The species has been the subject of numerous studies over the last few decades, most of which have concerned the striking abdominal colour and pattern polymorphisms found in virtually all populations (see Oxford and Reillo 1994 for full reference list). They are a strictly univoltine species but can occur in relatively high densities in meadows and at field edges (average of 18 m^{-2}) (Oxford and Shaw

1986), although the level of predation they apply to pest populations is currently unknown. Dispersal is thought to be limited to spiderlings, which may be passively dispersed by the wind when they are moving down from their natal leaves to the leaf litter where they overwinter (Oxford and Shaw 1986).

This relatively low level of dispersal, and hence limited potential for gene flow, means that populations can be postulated to exist in something akin to the isolation-by-distance model as outlined above, and allow a contrast between the genetic population structure of *L.tenuis* and *E.ovata* to be hypothesised - the former comprising of populations which have a high degree of inter-individual genetic variation at each site, but similar levels of variation between sites because of high levels of gene flow; the latter with individuals genetically similar at each site, but with differentiation between sites due to lower levels of gene flow and inbreeding.

1.2. Aims

To reiterate; whatever the causal factor(s) of dispersal, it is undoubtedly one of the major factors in the life history of *L.tenuis* and an attempt to quantify dispersal distances must be carried out if accurate predictions about population dynamics are to be successfully modelled. Dispersal ability greatly influences the population genetic structure of a species, and it is via the current genetic makeup of populations that dispersal distances of *L.tenuis* and *E.ovata* may be inferred. The main objective of the project is to address the following questions;

1) How is genetic variation distributed among populations of *L.tenuis* and *E.ovata*, and will this allow us to estimate the geographic boundaries of populations and hence effective dispersal distances?

2) Are there differences in the genetic diversity and differentiation between *L.tenuis* and *E.ovata* populations which are predictable in terms of their respective dispersal capabilities?

The first task was to identify the molecular tools best suited to answer these questions. There are many reviews of the techniques currently available to assess genetic variation (e.g. Burke *et al.* 1992; Hoy 1994; Mitton 1994; Karp *et al.* 1998) but it is useful to focus on the most salient.

1.3. Molecular marker systems

1.3.1. Allozymes

For some 30 years now, since the seminal work of Lewontin and Hubby (see Powell 1994 for a historical review), allozyme variation has been used as a tool to study gene flow and population structure in a large number of organisms. Allozyme differentiation is based on polymorphisms in non-denatured proteins which can be detected when they are size fractionated and specifically stained on a starch or cellulose gel. Allozyme band patterns are interpretable in terms of Mendelian genetic theory, and the allele frequency data from a population can be used to distinguish it from others using the statistical and genetic theorems of traditional population genetics, as propounded by Sewall Wright (1889-1988). Notably, the data for allozyme studies are population based rather than individual based. There is an

introduction to the general principles of allozyme genetic analysis in relation to spider taxonomy by Pennington (1979).

In terms of spider genetic studies in the literature, allozyme variation is currently the most travelled avenue onto population structure. Allozyme studies have posed many questions regarding population structure in relation to various aspects of the life history of spider species - the results often raising as many questions again!

Recently, for example, Rowell and Aviles (1995) studied sociality in the Australian huntsman spider *Delena cancerides* (Walckenaer), and found that despite high intercolony aggression, which should prevent interbreeding, a panmictic population structure appeared to exist, with solitary females hypothesised as the probable gene carriers between colonies. This confounds earlier work on the population structure of other co-operative social spiders. Roeloffs and Reichert (1988), working on *Agelena consociata* (Denis), detected genetic distinction between colonies as little as 30 m apart, whilst Smith and Engel (1994) carried out allozyme analysis on the population structure of the Indian co-operative spider *Stegodyphus sarasinorum* (Karsch) and found extreme subdivision between colonies (most colonies consisting of identical homozygotes). To contrast and compare with co-operative spiders, Steiner and Greenstone (1992) studied allozyme variation in five species of solitary spider, and found that genetic variation was four times higher than that observed in communal spiders. This is perhaps not unexpected, given the loss of heterogeneity which typically occurs in inbreeding organisms (Hartl and Clark 1997).

Ramirez and Fandino (1996) studied the genetic diversity of three populations of *Metepeira ventura* (Chamberlin & Ivie) on an island off the Californian coast using

a data set of 10 allozyme loci. Their results indicated an undifferentiated gene pool despite the patchiness of this species' preferential habitat. They inconclusively suggest that the results may either reflect genuinely low variability (perhaps due to a recent population bottleneck) or a greater level of dispersal than was anticipated between the favourable patches.

The foremost limitation of allozyme analysis in relation to invertebrate studies is that the allozymes of some invertebrate groups show markedly low variability. For example, the Homoptera have been quite extensively studied via allozyme analysis - Black *et al.* (1992), for example, reviewed the literature on many aphid species and found exceedingly low genetic variability. Similarly, low allozyme variability is notable throughout the Hymenoptera (see Graur 1985 for a review). As further, more pertinent examples of low allozyme variability, Roeloffs and Reichert (1988) reported that 15 of 22 (68 %) *Agelena consociata* allozyme loci studied were monomorphic; and Smith and Hagen (1996) found that only 7 of 40 (18 %) of the allozyme loci were variable in their study of the co-operative Theridion spider *Anelosimus eximius* (Keyserling), despite samples being many hundreds of kilometres apart. Smith and Engel (1994) summarised the allozyme work carried out up to that point on co-operative spiders and highlighted the generally low levels of polymorphism found. A further, more practical, limitation in identifying allozyme polymorphism may be the inability of conventional starch gel electrophoresis to detect amino acid substitutions, given that not all amino acid substitutions in a protein would change the electrophoretic mobility of the protein (nor indeed would all nucleotide changes necessarily produce amino acid changes!).

It must be remembered that by examining allozymes, a limit is immediately placed on the possibility of identifying variation, since only a fraction of the genome, in this case that coding for proteins, is being examined. Several DNA based "fingerprinting" techniques, in contrast, have the potential to assay the whole genome, both coding and non-coding regions, for variation and this advantage has led to an increasing number of studies employing fingerprinting techniques preferentially to allozymes. Stewart and Excoffier (1996), for example, state that a major advantage of analysing DNA fingerprint data is that it is generally a less biased estimator of genetic variation than gene-product level (i.e. allozyme) variation because of the whole-genome approach.

Finally, one practical problem that is often underestimated with allozyme analysis (whilst often used as a main avenue of criticism in DNA fingerprinting based studies) is the difficulty in setting up an allozyme system (buffer/stains) for each species under examination which delivers clear and, importantly, repeatable results (D.Rowell pers. comm.). Unless the researcher is fortunate to become involved with a group which has developed and optimised a system, many months can be wasted optimising different enzymes and running conditions.

As intimated briefly above, an alternative to examining proteins for variation is to examine the source of the protein variation, the genetic code itself, DNA. There are now a large number of techniques available which enable the researcher to examine DNA variation at a number of different levels of sophistication, each of which possess their own benefits and peccadilloes - some of the more salient methods will now be considered.

1.3.2. Sequence data

The ultimate level of DNA data acquisition is at the level of the nucleotide sequence i.e. the base composition (the building blocks) of DNA (N.B. it should be remembered that all other DNA based methods are essentially indirect attempts to gather sequence data). This has become a more practicable and accessible option with the advent of automated sequencing, which reduces the time required to generate data, and the polymerase chain reaction (PCR), which amplifies many copies of a given fragment of DNA facilitating its analysis (the PCR technique will be discussed in detail later in this chapter).

In the relatively recent past, due to cost and time factors, direct sequencing was usually reserved for phylogenetic studies, where fewer samples are typically required than may be the case for population based projects, and where high resolution accuracy is a necessity. Indeed, this has been the case for a number of the spider DNA based studies present in the literature. Gillespie *et al.* (1994) for example, sequenced a portion of the mitochondrial genome of several Tetragnathid species, and inferred multiple origins of the spider radiation in the Hawaiian archipelago; Huber *et al.* (1993), via sequence analysis of a portion of the 16s rDNA gene, have indicated the polyphyly of the Ctenidae family, and more recently, Piel and Nutt (1997), re-evaluated the phylogeny of the *Metepeira* genus, again using mitochondrial sequences.

Notably, with the greater ease of sequencing, a number of population level studies have been carried out using sequence data, including examples from within the Arachnida. Hedin (1997), for example, applied mitochondrial sequence data

generated from populations of sister species of cave spiders (*Nesticus* spp.) to address mechanisms of speciation. The results indicated that there was no correlation between differences in ecology and population structure, in contrast to previous allozyme based studies.

A single sequence on its own is naturally of little value to the researcher, but by examining the same regions from different species (or populations within a species) the study of molecular variation, population dynamics and systematics can be synergised.

In lieu of this high resolution approach to DNA analysis, a number of techniques have evolved which can generate sequence information about a fragment of DNA, but at a somewhat lower resolution.

1.3.3. DNA restriction analysis

Numerous studies have used restriction site diversity to infer population genetic structure. Indeed, some of the earliest analysis of DNA was based on the interpretation of DNA fragments produced by digestion of total genomic DNA using restriction enzymes. These enzymes, derived and purified from bacteria, restrict (cut) DNA, either total genomic, organelle, or fragments, when they encounter specific 4-, 5- or 6 base nucleotide sequences. For example, the enzyme *Eco*RI is a six base cutter, and restricts whenever it encounters the sequence 5'- GAATTC - 3'. This precise cutting can aptly be described as nature's exquisite scalpel. Polymorphisms in the sequence of nucleotides are then reflected by differences in the pattern of

restricted products (viewed following electrophoresis and staining). The digestion of highly repetitive sequences in the genome of many organisms generates relatively large numbers of fragments of DNA, which may give distinct patterns. However, this approach requires large amounts of genomic DNA, is time consuming, and in reality not particularly sensitive. An extension of this RFLP technique has proved much more effective for studying variation at a greater resolution.

Total DNA is digested as before, then blotted onto a nylon membrane (termed Southern blotting, after its first practitioner Dr. Edward Southern (1975)). A labelled probe (often radioactive) is then hybridised with the DNA on the membrane. The probe is specifically chosen by the user and can be almost any fragment of cloned or previously isolated DNA. Differences in banding patterns will reflect differences in the distribution of recognition sites for the restriction enzymes used in the original digest. These banding pattern differences are scored and used to estimate genetic parameters using statistical theorems. In relation to allozyme analysis, this is a more thorough assay of variability, as it will reflect differences throughout the genome i.e. both coding and non-coding regions. However, RFLP analysis is again time consuming and laborious, making it less viable for population based studies involving large numbers of individuals. Furthermore, the assay requires large quantities of relatively pure DNA, species-specific (homologous) DNA probes, and often uses radio-isotopes in the detection system - although recently non-radioactive labelled probes have become available e.g. digoxigenin from Boehringer-Mannheim, making this technique more accessible to laboratories without radioactive facilities (e.g. Neuhaus-Url and Neuhaus 1993).

1.3.4. DNA fingerprinting

DNA "fingerprinting" techniques have been widely adopted in many diverse fields of biology, including breeding and population genetics, evolutionary ecology, and forensic medicine. DNA fingerprinting, in its broadest sense, can be defined as any fine scale DNA analysis that allows the identification of samples to the level of the individual. The term has been specifically applied to analyses such as the studies of Variable Number of Tandem Repeats (VNTRs), Random Amplified Polymorphic DNA (RAPD), DNA Amplified Fingerprints (DAF), AP-PCR (Arbitrary Primed-PCR), PCR-RFLPs, microsatellites (also referred to as SSRs - Simple Sequence Repeats) and Amplified Length Polymorphisms (AFLPs), which will each now be briefly described. A wise molecular biologist quickly learns not to fear acronyms!

1.3.4.1. VNTR analysis

VNTR or "minisatellite" analysis, first described by Jeffreys *et al.* (1985a,b), is an RFLP based technique which uses a probe specific for a variable number tandem repeat (a locus of DNA, usually between 15-60 bp) which is repeated head-to-tail a variable number of times on a chromosome i.e. creating a DNA length polymorphism, as opposed to a sequence polymorphism. The technique is one of the most popular and reliable DNA fingerprinting methods, and is routinely used in forensic science, but can theoretically be applied to any population study. The length polymorphism is detected by hybridising a labelled probe (either minisatellite sequences, an entire hypervariable sequence, or synthetic oligonucleotides) to genomic DNA which has been digested with a restriction enzyme and Southern blotted to a nylon membrane.

The technique is very effective, but there are several limitations when applied to a study of the current nature. Whilst universal VNTR probes have been widely applied to vertebrates (humans in particular) and many probes are available, their application to invertebrates has been somewhat restricted and few studies are reported in the literature (e.g. Carvalho *et al.* 1991 is an exception to that rule). Thus it may be the case that species specific VNTR sequences would have to be cloned for each spider species studied, and this is extremely time consuming and technically demanding. Indeed, Hettle *et al.* (1997) highlighted that very sentiment, as they were unable to generate VNTR fingerprints from the spider *Pardosa pullata* (Clerck) using probes derived from available human sequences. Moreover, Southern blotting requires relatively large amounts of genomic DNA - potentially problematic for a small invertebrate species, and as such the VNTR option was not considered a viable option for this study.

1.3.4.2. The polymerase chain reaction

Whilst not a fingerprinting technique in itself, the analysis of nucleotide sequence variability, and hence the study of genetic polymorphism, was revolutionised in the late 1980s by the development of the polymerase chain reaction (PCR) (Mullis and Faloona 1987; Saiki *et al.* 1988). PCR allows the *in vitro* amplification of specific DNA sequences from minute quantities of starting material (theoretically a single molecule of DNA!). The production of many copies of a fragment greatly facilitates its subsequent analysis, be it observation on a gel, or the further steps of cloning and sequencing.

The basic reaction is reported in most biological text-books now, but bears repetition as it is possibly the single most important concept in current molecular biology. Indeed, it has been described, not necessarily with pathos, as the molecular equivalent of the invention of the wheel.

PCR amplification of a DNA fragment occurs in the presence of a polymerase enzyme, magnesium (a co-factor for the polymerase), and free dinucleotide triphosphates (dNTPs), which are the building blocks of DNA. A pair of oligonucleotide "primers", short synthesised single strands of DNA, usually 16-24 bases long, are designed to anneal to sequences directly flanking either side of the target sequence of DNA. Following denaturation of the double stranded template at high temperature, the temperature is dropped, allowing the primers to hybridise with their complementary sites on opposite strands of the target sequence. They are orientated so that DNA synthesis by the polymerase proceeds across the region between the primers. An exponential increase in the target DNA fragment occurs during the course of a repeated cycle of heat denaturation, annealing of the primers and extension of the annealed primers with incorporated dNTPs via DNA polymerase. The copy number increases exponentially due to the effective doubling of the extension product each cycle and its subsequent use as template itself.

A major, albeit perhaps the only, limitation of the process, is the requirement of sequence information to initially construct the primer pair. However, the application of so called "universal primers" which bind to and direct amplification of conserved i.e. homologous regions of DNA, across several taxa have proved

extremely useful in phylogenetic studies across a number of biological hierarchies (e.g. Kocher *et al.* 1989; Hillis and Dixon 1991).

1.3.4.3. PCR-RFLP analysis

The advent of PCR precipitated the PCR-RFLP technique, which utilises the power of PCR to amplify large quantities of specific sequences of DNA, which can then be assessed for variability by digestion with one or more restriction enzymes. By combining the power of PCR with that of restriction enzymes highly sensitive assays are possible. The two most commonly used genomic targets are multi-copy ribosomal DNA and mitochondrial DNA fragments, both of which can be readily amplified by "universal primers" (Hillis and Dixon 1991; Kocher *et al.* 1989 respectively) - although single copy nuclear loci have also been employed e.g. Karl and Avise (1993).

Mitochondrial DNA is often chosen for PCR-RFLP studies for several reasons. The mitochondrial genome evolves approximately 5-10 times faster than single copy nuclear genes, whilst also retaining conserved regions allowing "universal primer" PCR amplification. Additionally, many mitochondria are present in most tissues, presenting a large number of target molecules. Dawson *et al.* (1995), for example, used PCR-RFLP of the V7 region of the small mitochondrial RNA gene to differentiate between sub-populations of the tree *Gliricidia sepium*. Importantly, mitochondrial DNA is maternally inherited and therefore effectively haploid. This results in a reduction in the effective number of genes when males and females are present in equal numbers (in contrast to bi-parentally inherited genes). These

properties - high mutation rate and maternal inheritance - are valuable for analyses of population structure in that they tend to increase the proportion of variability distributed among populations and allow for a more rapid sorting of ancestral alleles within and between species. These factors, coupled with the lack of segregation and recombination within mtDNA, means that the mitochondrial genome contains complete and unambiguous information, and studies have produced information on the lineages and population structure of both vertebrates (e.g. Caan *et al.* 1987, who traced mtDNA lineages to a hypothetical African "mitochondrial Eve" in our own species) and invertebrates (e.g. Boyce *et al.* 1989; Hall and Smith 1991 studying Bark Weevils and honey bees respectively). However, a recent paper by Zhang and Hewitt (1996) casts doubt on the future unrestricted use of PCR amplified mtDNA fragments from total cellular DNA in intraspecific population studies. Following ambiguities in their sequence data they investigated further and detected the presence of highly conserved non-coding mitochondrial sequences in the nuclear genome of *Schistocerca gregaria*, the desert locust. This highlights that ambiguous polymorphisms can be present, which reflect not true mitochondrial diversity, but differences between true mitochondrial and nuclear incorporated sequences. The paper concludes that a preliminary screen for nuclear copies of the mtDNA sequence in question should be carried out - adding a further level of complexity to the procedure.

Ribosomal DNA has also been extensively utilised for species and population level studies, as it is also ubiquitous, and contains a number of DNA elements which evolve at different rates, allowing a number of biological hierarchies to be assessed

dependent on the needs of the current study. Stothard *et al.* (1996) for example, used six restriction enzymes to digest the internal transcribed spacer region of the snail genus *Bulinus* and found considerable variation between species - indeed, sufficient to question the placement of some of the species within the same genus; whilst Vogler and DeSalle (1994) reported high levels of sequence polymorphism in the internal transcribed spacer (ITS) region of populations of the tiger beetle *Cicindela dorsalis*. The potential utility of rDNA for population studies of arthropods has been discussed (Roderick 1996) and will be addressed in detail in Chapter Five.

1.3.4.4. RAPD-PCR analysis

A development of PCR, random amplified polymorphic DNA (RAPD) analysis, overcame the problem of requiring prior sequence information for primer construction. The technique, described by Williams *et al.* (1990), uses only a single short primer (usually 10 bases in length) which hybridises with complimentary regions of the genome. The sequence for the primer is simply chosen at random (although usually containing 50-60 % GC content), thus no prior knowledge of the target genome is necessary. Furthermore, because the reaction relies on the amplification process of the polymerase chain reaction, only relatively small quantities of template DNA are required. Indeed, consistent RAPD profiles have been produced from single juveniles of *Globodera* spp. nematode (Roosien *et al.* 1993) and from minute microhymenopteran species (Landry *et al.* 1993).

RAPD analysis is one of a number of independently developed random priming fingerprinting strategies which have collectively been termed multiple

arbitrary amplicon profiling (MAAP) (Caetano-Anolles *et al.* 1992). AP-PCR and DAF are two alternatives which work on much the same principle as the RAPD technique but with a few minor differences. AP-PCR, for example, again utilises a single random primer, but primer length is comparable with specific PCR (16-30 bases). A second difference is that the annealing temperature is raised following the first few cycles of amplification, to increase the specificity of the reaction e.g. Fukatsu and Ishikawa (1994). The DAF method employs primers considerably shorter than those used in RAPD analysis (5-8 bp), but in this case, the thermal cycling has comparable annealing temperatures (e.g. Caetanno-Annoles 1993). Each DNA fingerprinting method generates a characteristic type of profile - DAF amplification products are separated by necessity on polyacrylamide gels because of the complexity of the profile obtained (the very short primers find many annealing site across the genome) - whereas the fewer bands produced via RAPD and AP-PCR profiles can typically be resolved in agarose gel. A review of the literature indicates that RAPD analysis is by far the most widely practised MAAP technique, probably due to the positive trade-off between the number of bands per lane against the ease of resolving the amplification products via agarose gels and ethidium staining. Chapter Four examines the RAPD technique in detail.

1.3.4.5. Microsatellite analysis

A further high-resolution fingerprinting technique which is rapidly growing in popularity is that of microsatellite analysis. Microsatellites, also known as Simple Sequence Repeats (SSRs), are tandemly repeated short motifs of DNA between 2-5 bp in length (e.g. $(CG)_n$ or $(CGG)_n$) that occur in abundance throughout the genome of many organisms, including insects (e.g. Ender *et al.* 1996). As these repeat units are readily added to or lost from the SSR region, the length of these regions evolves rapidly, generating detectable polymorphisms.

SSRs are generally amplified via PCR using a primer pair which flank a particular repeat region - with any length differences visualised through electrophoresis on a polyacrylamide gel - and are thought to offer a valuable pool of high resolution genetic variation and is considered particularly useful when other methods (e.g. allozyme analysis) show insufficient variability. Generally, SSRs are codominant (in contrast to RAPD fragments), reveal polymorphic amplification products from all individuals in a population, and allow the elucidation of the zygotic state of the individual.

The main draw-back with this method is the effort and expertise required to initially identify and clone SSRs from a genomic library of the chosen organism. This step may be omitted, however, if primers previously found to work on a related species can be applied. For example, Coote and Buford (1997) recently published SSR primers which are applicable across both apes and Old World monkeys. Unfortunately there are no spider SSR loci currently available in the literature.

Zietkiewicz *et al.* (1994) introduced a method for applying anchored SSR primers, in essence acting as random primers. This method involves synthesising primers containing a repeat motif with "anchoring" bases either at the 5' or 3' end e.g. $(CA)_nNN$ or $NNNN(CA)_n$. The primers are used one at a time, and will amplify genomic sequences flanked by two inversely orientated $(CA)_n$ elements. The anchored bases means that the primer will bind at the ends of a repeat regions and not internally. Thus the anchored primer may be used as a "random" fingerprinting marker, or used to highlight microsatellite sequences for further characterisation.

Both standard PCR and anchored PCR were critically evaluated in relation to amplifying SSR regions by Weising *et al.* (1995). Interestingly, despite its increasing use as perhaps the fingerprinting method of choice over the last few years, they reported similar difficulties using both single locus microsatellite primers and anchored microsatellite primers as are encountered with RAPD amplification, viz, the influence of slight changes in PCR protocols and conditions on the repeatability of banding patterns produced.

1.3.4.6. AFLP analysis

Parallel with the evolving nature of DNA itself, is the continual development of new methods to detect genetic variation. The AFLP approach to genome analysis (Vos *et al.* 1995) combines features from several of the previously discussed methods, and is fast gaining popularity due to the high number of markers it can generate. With appropriate conditions, more than 100 loci per gel lane can be scored, a large proportion of which may be polymorphic between genomes (Rafalski *et al.* 1996). The technique is based on the selective PCR amplification of restriction

fragments of a genomic digest. Sharma *et al.* (1996) carried out a comparative study on the diversity and phylogeny of lentils using both RAPD and AFLP techniques, and concluded that although both techniques provided similar conclusions, AFLP detected a much higher level of polymorphism. They concluded that the greater level of variation detected makes it a more efficient marker technology than RAPD-PCR. However, the use of radioactivity in the detection system and the number of steps required to produce data make this technique relatively demanding in terms of both equipment and technique.

Table 1.2. Summary of a selection of DNA based marker systems

	RFLP	RAPD	Micro-satellites	AFLP	PCR-RFLP	Sequencing
Principle	Restriction endonuclease digestion, Southern blotting, hybridisation	DNA amplification with random short primers	PCR of simple repeat regions	PCR of a subset of restriction fragments	Restriction digestion of PCR fragments	PCR, cloning, manual or automated sequencing
Nature of polymorphism	Single base changes, insertions, deletions	Single base changes, insertions, deletions	Repeat length changes	Single base changes, insertions, deletions	Single base changes, insertions, deletions	Base composition
Genomic abundance	High	Very high	High	High	High	Total
Level of polymorphism	Medium	Medium	High	Medium	Medium	Potentially very high
Dominance	Codominant	Dominant	Codominant	Mixed	Codominant	N/A
Multiplex ratio*	1-2	5-20 (adjustable)	1	30-100, (adjustable)	Low	N/A
DNA amount required	2-10μg	10-25ng	50-100ng	1-2 μg	50-100ng	10-100ng (for PCR)
Sequence information required?	No	No	Yes	No	Yes	Yes
Radioactive detection	Yes/no	No	No/yes	Yes/no	No	Yes/no
Start up and development cost	Medium-high	Low	High	Medium	Medium-high	High

* The multiplex ratio is a term adopted to describe the average number of genetic loci that may be simultaneously analysed per gel lane (From Rafalski *et al.* 1996).

1.4. Summary

As highlighted in Table 1.2, the last decade has seen the development of DNA based techniques which enable researchers to examine genetic variation in ever finer detail, down to the level of nucleotide sequence itself. The ability to study organisms at this level, in conjunction with more traditional population dynamic data, is potentially of great benefit to researchers in answering previously intractable questions in a host of ecological fields. The various levels of sensitivity the marker systems provide however, presents an important challenge to the researcher, who must carefully choose the technique most appropriate to the study in hand, in terms of balancing the resources and equipment available, against the potential benefits of each technique.

For the purposes of this study, to generate information on population variation which could then be analysed in terms of population structure, it was decided to carry out two PCR based techniques with spider DNA. Firstly, RAPD analysis would be carried out to gather genetic information on populations at a relatively local level (< 100 km). As discussed, RAPD amplification does not require prior sequence information, of which there is scant available for spiders, and is reported by many authors as a user-friendly, technically undemanding technique which can readily detect relatively high levels of polymorphism. This made RAPD analysis an appealing choice.

Secondly, an analysis of mitochondrial and ribosomal DNA would be carried out with populations from a wider geographic range, not only to assess levels of genetic variation *per se*, but also to allow the investigation of the phenomenon of

concerted evolution in spider rDNA, via analysis of the internal transcribed spacer regions (ITS) (the concept of concerted evolution is introduced and discussed in detail in Chapter Five).

The analysis will be carried out in two stages; following PCR amplification of potentially variable regions, PCR-RFLP will be carried out as an initial screen for population specific polymorphisms. The sequencing of variable regions would then enable analysis of genetic variation at the finest level possible.

To my knowledge, no DNA based studies have been carried out on either *L.tenuis* or *E.ovata*. Indeed, there are few studies of the population dynamics of invertebrate predators, and few studies on migratory species, mainly because of the methodological problems involved in quantifying migration (Sunderland and Topping 1993). The beneficial nature of spiders, combined with the paucity of basic population genetic information currently available, make this a fascinating area in which to carry out research.

Chapter Two presents the pilot experiments undertaken whilst optimising the chosen techniques for spider analyses.

2. DEVELOPMENT OF PROTOCOLS FOR THE EXTRACTION, STORAGE, AND PCR AMPLIFICATION OF SPIDER DNA

2.1. Introduction

As there is limited literature regarding spider DNA based studies, a review was undertaken to examine relevant protocols for invertebrate specimen storage regimes, DNA extraction methods, and PCR amplification. To utilise PCR based methods a reliable DNA extraction method which generates high quality DNA, and is relatively fast (allowing the processing of the number of individuals necessary for a population survey), is paramount and this issue was addressed as a priority in the laboratory. A number of other factors such as spider storage pre-DNA extraction and the subsequent storage of extracted DNA were also addressed and optimised.

Protocols for RAPD-PCR and specific PCR were then optimised and important factors such as the reproducibility of RAPD profiles, band homology and the potential of DNA contamination addressed. Furthermore, the adaptability of the optimised RAPD protocols was highlighted by generating profiles from three spider families and from second instar spiderlings. A condensed version of these protocols are presented in the research note of A'Hara *et al.* (1998).

2.2. Storage prior to DNA extraction

The literature on the most appropriate storage of invertebrates pre-DNA extraction is far from helpful and often contradictory, particularly in terms of the benefits of storage in ethanol. As a preliminary experiment to elucidate the effect of storage conditions on the quality of spider genomic DNA, whole spiders were stored

in three different ways; in 70 % ethanol at room temperature in the dark (8 to 12 ^{o}C); in ethylene glycol at room temperature in the dark, or frozen in liquid nitrogen and stored at -80 ^{o}C.

2.3. DNA extraction, quantification and subsequent storage

2.3.1. DNA extraction

Two DNA extraction methods were initially considered, to ascertain which would be the more appropriate for this study. Firstly, extractions were carried out following the standard phenol/chloroform solvent extraction method as described in detail in Sambrook *et al.* (1989) with little or no modification, although liquid nitrogen was used to snap freeze the sample prior to homogenisation with a plastic Eppendorf pestle, as freezing aids the complete destruction of tissue.

Secondly, a DNA extraction method modified from the Cheung *et al.* (1993) protocol, designed for the rapid extraction of DNA specifically for PCR based analysis, was assessed. As this became the extraction method of choice a detailed description of the procedure follows.

During the extraction procedure all samples were kept on ice to prevent degradation of the DNA by endonucleases which are released from tissue following thawing. A 1.5 ml Eppendorf tube containing an adult spider was lowered into liquid nitrogen for 10 seconds then the spider tipped out onto a Petri dish lid. The abdomen was removed with a sterile scalpel blade (flamed between specimens), thereby preventing the possible amplification of DNA from prey ingested by the spider and present in the digestive mass, or of parasitic larvae (suspected to be Hymenoptera),

which were occasionally observed to attach to the abdomen, but never the carapace. The carapace was then immediately returned to the tube and refrozen (notably, this protocol has the benefit of taking place in only two tubes per specimen, in contrast to the phenol/chloroform extraction which requires several tubes per sample).

The frozen tissue was homogenised with a sterile plastic Eppendorf pestle (a separate pestle was used for each spider to prevent DNA cross contamination), then 500 µl chilled DNA extraction buffer (200 mM Tris-HCl (pH 8.0), 70 mM EDTA, 2 M NaCl, 20 mM sodium metabisulphite) and 90 µl of a 5 % w/v N-lauryl sarcosine (Sigma, UK) (which assists in the lysing of cells) were added, and additional grinding carried out to ensure complete destruction of tissue. If additional pestles were required, the end of a 1 ml Gilson pipette tip was melted with a Bunsen burner and quickly pressed into a 1.5ml Eppendorf tube. By rotating the tip whilst the melted plastic set, a tight fitting pestle is created. After grinding, the tubes were then incubated at 65 ^{o}C for 1 hour to both lyse the cells and denature endonucleases. During incubation, the tubes were gently mixed by occasional inversion.

Following incubation, the homogenised tissue was spun in a microfuge at 12,000 rpm for 3 min to pellet gross cell debris, and the supernatant, containing the DNA, transferred to a fresh labelled tube. To precipitate the DNA, 90 µl of 10 M ammonium acetate and 400 µl of chilled 100 % isopropanol were added to the supernatant, the tube slowly inverted several times to mix, and the sample placed at -20 ^{o}C for a minimum of two hours, but typically overnight.

The precipitated DNA was pelleted at 12,000 rpm for 12 min, after which the supernatant was poured off and 400 µl of 70 % ethanol added. The Eppendorf tubes

were turned round in the centrifuge rotor at this point (i.e. hinges now pointing in) to ensure the pellet moved through the 70 % ethanol when spun. This washing step is important in removing any remaining PCR inhibitors present in the pellet. By forcing the pellet through the alcohol, rather than just washing the surface of the compacted pellet, this ensures the step is maximally effective. Following a 5 min spin, the 70 % ethanol was decanted.

Finally, the pellet was air dried for 30 to 45 min in a laminar flow cabinet by inversion of the Eppendorfs on tissue paper. It is critical to ensure all the ethanol has evaporated as alcohol can negatively affect PCR amplification (R. Finch pers. comm.). However, over-drying can also lead to problems re-dissolving the DNA, so a balance must be reached. The dried pellets were subsequently resuspended in 50 µl of either PCR-grade sterile water (Sigma, UK), or TE (10mM Tris-HCl (pH 7.5), 0.1mM EDTA) buffer, to allow a comparison of which was superior for storage prior to amplification. DNA resuspension was aided by heating at 60 ^{o}C for 1 hour.

2.3.2. DNA quantification

DNA extractions were quantified as described in Section 3.3.3.

2.3.3. DNA storage for subsequent RAPD amplification

During these preliminary experiments, a proportion of the extractions were treated with the nuclease RNase A (5 µl of a 10 mg / ml dilution; Pharmacia, UK) to digest any RNA present post-extraction, allowing us to observe if this improved the RAPD profiles subsequently generated. DNA was then stored in 10 µl aliquots at either 4 °C (both undiluted and diluted for PCR (typically 1:10 with SDW)) or at -20 °C (undiluted) until utilised in a RAPD reaction, thus allowing the evaluation of the best storage temperature post-extraction. Primers OP-AR19 (5'-CTGATCGCGG-3') and OP-B03 (5'-CATCCCCCTG-3') were chosen at random from those available in the laboratory and used in RAPD-PCR reactions with both the aliquoted "neat" DNA (which was diluted to working concentration just prior to PCR), the DNA held at 4 °C already diluted to the 1:10 working concentration for PCR, and dilutions of the "neat" DNA held at -20 °C. PCR amplifications were carried out at intervals of one week, two weeks and one month, to test the reproducibility of the profiles over time.

2.4. RAPD-PCR optimisation

No RAPD studies on spiders had been reported in the literature prior to undertaking this study. However, researchers employing the RAPD technique have highlighted the benefits of optimising protocols for the particular organism/primer combinations used, and hence a number of variations and conditions were attempted in pilot experiments to optimise the protocol for this study.

DNA amplification was carried out primarily on a Perkin Elmer TC-1 thermal cycler. Three cycling regimes were evaluated; consisting of either 35, 40 or 45 cycles

of 1 min at 95 °C for DNA denaturation, followed by 1 min at 36 °C for primer annealing, and 2 min at 72 °C for primer extension. This was typically preceded by a DNA denaturation step of 2 min at 95 °C, although, again, experiments were carried out with varying denaturing times. The cycling was always followed by a final primer extension step at 72 °C for 7 min to ensure completion of the amplified fragments. A second PCR machine, the Techne Genepro thermal cycler, was also briefly tested to compare results (a worthwhile comparison as different machines are reported to be the major factor in differences in RAPD profiles generated during inter-lab studies using identical DNA samples (e.g. Rafalski *et al.* 1996)).

Optimisation was carried out with a range of magnesium chloride concentrations (1-5 mM) and a range of DNA template dilutions (approximating to 1-100 ng) to discern which combination would produce the best results (defined subjectively as crisp and clear banding patterns). These two components were given particular attention as they are held by many authors (e.g. Bassam *et al.* 1992) to have the greatest effect on profile quality (magnesium is a co-factor for the polymerase enzyme and is hence vital for its function). The primer (10-base primers, Operon Technologies Inc., USA), dNTP and *Taq* polymerase concentrations were as described by Williams *et al.* (1990). *Taq* enzyme, magnesium chloride, polymerase buffer and dNTPs were supplied by Perkin Elmer, UK. Initial experiments were carried out using both Stoffel *Taq,* a truncated form of the polymerase enzyme, and AmpliTaq *Taq*, to compare proficiency of amplification and clarity of profiles (the former has reputed advantages for DNA profiling).

A negative control, comprising the above reaction mix, but with water replacing DNA template, was included with each PCR run to preclude the possibility of any bands present having resulted from contamination in any of the PCR mix components.

On a purely practical note, visual confirmation of the delivery of all reagents was made whilst setting up any PCR reaction. This may seem a moot point, but when dealing with the small volumes of reagents typical in this PCR work, observing this rule can be of considerable importance. A further practicality was that the magnesium, polymerase buffer, dNTPs, primers and PCR water (Sigma, UK) were all aliquoted on arrival in the laboratory. This reduced the possibility of contamination and prevented the reagents undergoing multiple freeze thaw cycles, which is generally not recommended.

Finally, prior to thermal cycling, the reaction mix was overlaid with approximately 25 µl of light mineral oil (Sigma, UK) - one drop from a 1 ml Gilson pipette tip, to prevent evaporation and reflux of the sample during cycling.

2.4.1. Adaptability of the RAPD technique

The main strength and utility of the RAPD technique, and indeed the majority of DNA based techniques, is the ability to transfer to any chosen genome with the minimum of problems, given the constancy of the genetic code. The second major benefit of RAPD analysis is that the power of PCR allows the amplification of DNA sequences from very small starting quantities of DNA. These two premises were

tested early in the course of this study via two experiments which aimed to indicate the value and adaptability of the RAPD technique to arachnological studies.

2.4.1.1. Amplification from three spider families

Three primers were chosen: OP-B03 (5'-CATCCCCTG-3'), OP-AF15 (5'-CACGAACCTC-3') and OP-AR19 (5'-CTGATCGCGG-3'), and tested with five individuals from three spider species drawn from three families: *L.tenuis* (Linyphiidae); *E.ovata* (Theridiidae); and *Clubiona reclusa* (O.P.-Cambridge) (Clubioniidae).

2.4.1.2. Amplification of spiderling DNA

As a second test of adaptability, RAPD-PCR was carried out with *L.tenuis* spiderlings and their respective mothers, to gauge the feasibility of using this protocol with immature spiders. Primers OP-B03 (5'-CATCCCCTG-3'), OP-AF15 (5'-CACGAACCTC-3') and OP-AR19 (5'-CTGATCGCGG-3') and OP-H2 (5'-TCGGACGTGA-3') were used to amplify DNA from three mothers and their offspring. DNA was extracted basically as presented in Section 2.3, although increased care was required when homogenising the spiderling to ensure all tissue was washed off the pestle by the extraction buffer, as it was easy to inadvertently remove the sample from the tube still adhering to the pestle. Also the pellet was resuspended in only 20 µl SDW of which 4 µl was used per RAPD reaction.

2.4.2. Homology of RAPD bands

RAPD analysis is based on scoring for the presence or the absence of amplified DNA fragments separated on a gel. For the analysis to be an accurate measure of genetic similarity, fragments which appear to be homologous in size on a gel should actually be homologous in terms of their base sequence, and not simply similarly sized fragments of different genomic origin. It would be possible to excise, gel purify, then sequence and compare all RAPD fragments, but this is an expensive and time consuming option. A second, more realistic option to test the homology of RAPD products is to gel purify, concentrate, then restrict specific fragments and compare the restriction profiles. A similar profile would be indicative of sequence homology. A small scale experiment of this nature was carried out.

RAPD reactions were carried out with four *E.ovata* individuals from two sites, Edinburgh and Invergowrie, with primer OP-AR19 (5'-CTGATCGCGG-3'). The amplification products were run on a 1 % low melting point (LMP) agarose gel and in each case a clearly defined bright band (approximately 1000 bp in size), showing apparent homology, was pierced with a 1 ml Gilson pipette tip. The gel plug was expelled into an Eppendorf tube containing 20 µl SDW, then placed in a heating block at 80 ^{o}C until the gel dissolved fully (approximately 10 min). Two microlitres of this DNA solution was then used as template for re-amplification with the original primers. Eight µl of the resultant PCR was run on a 1.5 % agarose gel to assess if re-amplification was successful (i.e. a single band of the original size).

Unfortunately, despite all reasonable precautions, several smaller bands were consistently found in the PCR product following re-amplification. Possibly the bands were due to nested primer sites (the presence of repeated primer sites within the main product itself), or perhaps contamination was occurring. Nevertheless, a further purification step was found to be necessary.

Twenty microlitres of the original RAPD product were again electrophoresed on a 1 % agarose gel and the band excised with a scalpel. DNA was then extracted from the gel slice by using a DNA extraction kit (Sigma, UK) following the manufacturer's instructions, and resuspended in 24 µl SDW. This was then split into three aliquots and digested overnight with restriction enzymes *Eco*RI (6 base cutter), *Dde*I (5 base cutter), and *Hae*III (4 base cutter). The digested fragments were electrophoresed on a 2 % agarose gel at 80 V for two hours and stained with ethidium bromide prior to UV illumination.

2.4.3. Screening for potential contaminants

2.4.3.1. Comparison of RAPD profiles from abdomen and carapace

As previously emphasised, the main strength of PCR lies in its ability to amplify DNA from minute quantities of starting material. This ironically leads to the possibility that DNA from exo- and endosybionts present on, or in, the organism of study may be co-amplified (or indeed, solely amplified!), giving a mis-leading profile (e.g. Fenton *et al.* 1994; Bidochka *et al.* 1997). To allay fears of contamination, experiments were carried out to directly compare RAPD profiles generated from DNA extractions from the abdomen and carapace of several individuals, to gauge the

extent of any spurious bands which may be present due to non-target contamination or parasite burden.

2.4.3.2. Screening for microbial contaminants

Secondly, an experiment was carried out to ascertain the level of exosymbiont fungal or bacterial contamination of samples and whether these would affect amplification profiles. Ten unidentified linyphiids were placed in 1 ml of sterile water with a drop of 20 % Tween detergent, and gently shaken. A serial dilution (10^{0}-10^{-4}) of the resulting wash, (which would contain spores of any potential contaminant) was plated onto Potato Dextrose Agar and Nutrient Agar plates (Sigma, UK prepared as per manufacturer's instructions), which would provide suitable substrates for most micro-organisms.

Two main contaminants were recorded. *Sporobolomyces* yeast, identified by its distinctive orangey colour and consistency, and a sterile white fluffy mould, were both found to be growing (R. Harling pers. comm.). A loopful of each colony was transferred separately to a PCR tube containing 100 µl DNA extraction buffer and heated for 15 min at 95 ^{o}C, lysing DNA from the cells. Five microlitres of this lysate was used directly as the template DNA in RAPD reactions with two primers: OP-AR13 (5'-GGGTCGGCTT-3') and OP-AR18 (5'-CTACCGGCCAC-3'). Simultaneously, a RAPD reaction was carried out with the same primers and bulked DNA extracted from five *L.tenuis*, to allow a comparison of spider and putative contaminant profiles.

2.5. Amplification with universal primers

2.5.1. Amplification of mitochondrial DNA

Insect-specific primers for conserved regions of the cytochrome oxidase I (COI) and cytochrome oxidase II (COII) mitochondrial genes, flanking a potentially polymorphic intervening region were used in an attempt to amplify this region from spider DNA (Roehrdanz 1993):

COI-RLR 5'-TTGATTTTTTGGTCATCCAGAAGT-3' (24 bases).

COII-Croz 5'-CCACAAATTTCTGAACATTGACC-3' (23 bases).

These primers were chosen due to their availability in the laboratory and the belief that DNA conservation within the Insecta would facilitate amplification of the same region in the Arachnida. The primers amplified a 1400 bp fragment from aphid genomic DNA. A range of annealing temperatures (between 35-60 ^{o}C), magnesium (1-6 mM), and DNA (1-100 ng) concentrations were used in an attempt to optimise the PCR amplification.

2.5.2. Amplification of ribosomal DNA

To amplify spider rDNA fragments, specific primers and reaction conditions were initially employed as reported by Fenton *et al.* (1997) and are reported in detail in Chapter Five.

2.6. RESULTS AND DISCUSSION

2.6.1. Pre-extraction storage of spiders

Despite maintaining the physical integrity of the specimen, the DNA extracted from spiders stored at room temperature in 70 % ethanol was found to have degraded completely after three weeks (Figure 2.1a). Storage in ethanol for future DNA extraction is something of a contentious issue, with reports ranging from animal tissues stored for six years producing high yields of high molecular weight DNA (Smith *et al.* 1987), to Coleopteran DNA which maintained its integrity for only six weeks in 95 % ethanol (Reiss *et al.* 1995). Storing in ethanol at 4 ^{o}C may perhaps have improve the longevity of DNA integrity, as lower temperatures inhibit endonucleases which break down the DNA, but nonetheless storage in alcohol is problematic. A discussion amongst Bug-net users (on-line newsgroup "bug-net@sfu.ca" dealing with invertebrate population genetic issues) prompted a number of observations on the preservation of invertebrates for future molecular studies. Consensus was hard to achieve, but absolute alcohol (100 %) storage for short term storage (days rather than weeks) was thought acceptable - although care must be taken to dry the specimen thoroughly prior to extraction. Even then, amplifying fragments of > 1000 bp in size were reportedly rare. The findings in this study are in accordance with those views, as samples sent from New Zealand in absolute alcohol (approximately ten days shipping) were found to contain mildly degraded DNA from which about 60 % of amplifications were successful for a 1200 bp fragment. Laulier *et al.* (1995) state that DNA can be recovered from ethanol and methanol preserved

samples, but the degree of degradation appears to be species specific, and the yield is generally poor. It can be speculated that any species specificity of degradation may be due to the physical properties of the cuticle of the organism. The report of a six week period prior to degradation for Coleopteran DNA may be indicative of the hard exoskeleton preventing the alcohol reaching the softer tissues as quickly as was found to be the case with spiders. Other possibilities remain. Zang and Hewitt (1998a) for example, are strongly convinced that associated gut flora plays a role in degrading DNA. They found that starving beetles for one week prior to preservation in alcohol lead to a good DNA yield - in stark contrast to beetles put into alcohol directly.

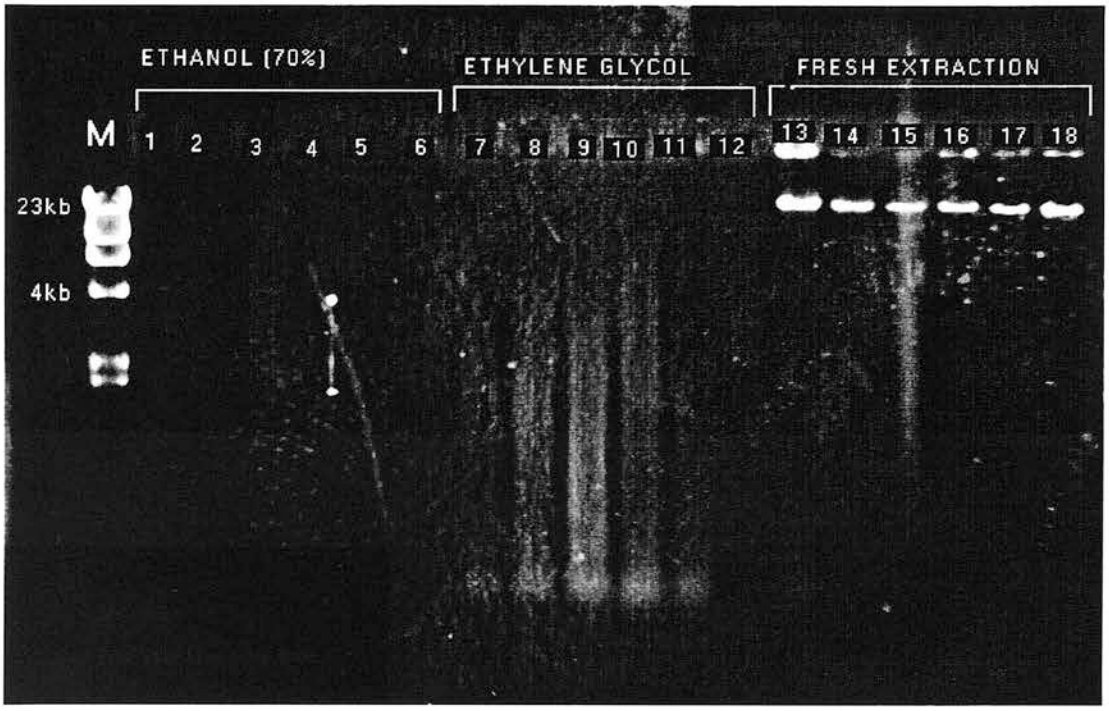
Finally, Ito (1992) reports that unknown contaminants present in even 100 % ethanol can cause degradation of DNA. This ultimately led the author to the simple classification of ethanols as "good" and "bad"! The findings of this study support the difficulty of finding a "good" ethanol and it may be prudent not to take the risk if possible of returning to specimens only to discover the DNA is degraded to a point where it cannot be used. Therefore, ideally, an alternative to alcohol preservation should be used.

Ethylene glycol, commonly used in pitfall traps as a preservative, also appears to degrade DNA across a similar timescale, despite preserving specimens morphologically for at least a year and therefore cannot be recommended for samples which will undergo future DNA analysis. A paper by Frey and Frey (1995) reported that Tanglefoot™, a sticky glue commonly used with aerial traps, preserved the DNA in scale insects adequately for RAPD identification. If further spider studies are undertaken this would seem a method particularly worth following up, given the

ballooning frequency of *L.tenuis*. Furthermore, a saturated salt solution has been assessed by several researchers in field studies and is thought to preserve the DNA in samples for up to several months without refrigeration, and even longer if stored at 4 °C. Again this method was only discovered latterly in the course of this study and was not investigated, but would seem highly applicable in overcoming problems associated with invertebrate collections (e.g. a solution that could be left in a pitfall trap for several days prior to emptying the trap which would preserve DNA (cf. ethylene glycol) would be highly advantageous).

Ultimately, however, for ease and speed, specimens frozen at -80 °C were found to produce excellent high molecular weight DNA extractions for at least 12 months after freezing (Figure 2.1b), and this was chosen as the storage method of choice for whole spiders prior to DNA extraction. Notably, it was necessary to identify the spiders prior to freezing, as the delicate tissues of the sexual organs (epigyna and palps) darkened considerably following freezing and made identification problematic. The main problems with storage at low temperature are the premium for space, (with careful packing and choice of container, many hundreds of samples can be held in very little space) and the potential of freezer failure, which unfortunately did occur during this study.

(a)



(b)

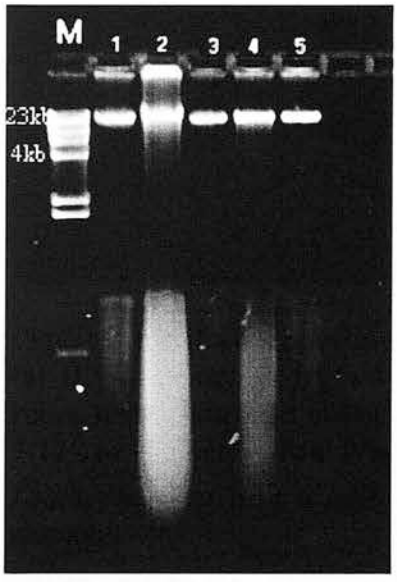


Figure 2.1. Effect of specimen preservation on genomic DNA.
(a) DNA extraction from 18 *Lepthyphantes tenuis*, stored for three weeks at room temperature in either 70 % ethanol (lanes 1-6) or ethylene glycol (lanes 7-12), or extracted from fresh tissue (lanes 13-18) (b) DNA extraction from five *Lepthyphantes tenuis* stored at -80 ^{o}C for 12 months. M= high molecular weight marker.

2.6.2. DNA extraction, quantification and storage

2.6.2.1. DNA extraction

The modified Cheung *et al.* (1993) extraction protocol consistently yielded greater than 5 μg of DNA from each individual *L.tenuis* carapace, and at least double for the larger *E.ovata* and *C.reclusa*, although the actual quantity could vary from individual to individual (Figure 2.2). Notably, the quantity of the DNA recovered was comparable with DNA extracted using the more traditional, and time consuming, solvent extraction method (results not shown), but avoided the unpleasantness of handling phenol and chloroform. In addition, the fact that the entire extraction procedure takes place in two tubes also limits the potential for mis-labelling when a large number of tubes are being handled simultaneously. On a purely practical level, 16 individual spiders were processed at a time, as a balance between throughput and the amount of time the samples were out of the -80 °C freezer.

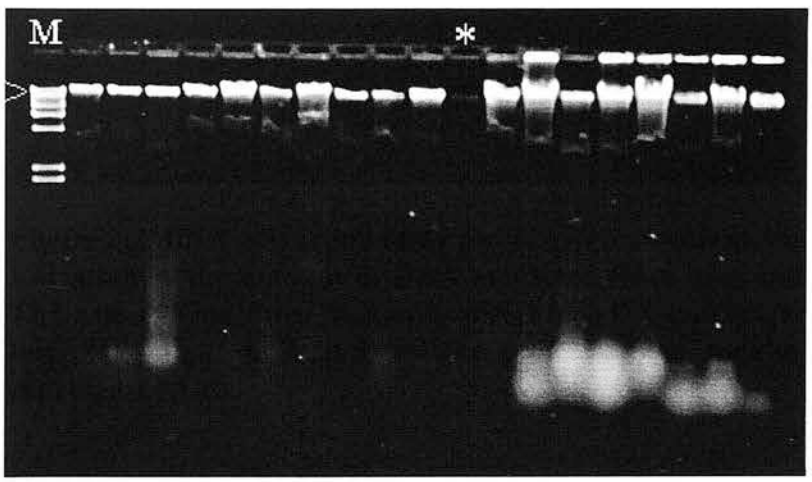


Figure 2.2. DNA extraction of 19 *Enoplognatha ovata* specimens.
Variation in the amount of DNA extracted from each individual can be seen. The asterisk indicates a sample where the DNA pellet has been accidentally lost. M= high molecular weight marker. The highlighted marker band represents 23 kb.

It is worth emphasising that an effective DNA extraction and storage regime is one of the most vital aspects of PCR analysis. A common misconception regarding the RAPD technique - created due to the mystique surrounding the ability of PCR to rescue archival fragments of DNA - and one which must be laid to rest, is that poor quality DNA can be utilised and meaningful, reproducible results achieved. This is simply not the case. Forensic or archival DNA material which is PCR amplified typically consists of very small fragments of high copy number sequences of DNA, such as mitochondrial control regions (< 400 bp) amplified using specific primers (Refer to Paabo *et al.* (1989) for an example of the recovery of ancient DNA). RAPD profiling, which is amplifying theoretically from the entire genome, requires undegraded DNA to produce a clear repeatable profile. This has been painfully illustrated by an inability to obtain profiles from samples which were being held at -80 ^{o}C, prior to DNA extraction, which accidentally defrosted due to freezer failure. The DNA recovered was highly sheared when electrophoresed and viewed on an agarose gel, but high molecular weight DNA was present. However, despite much effort, RAPD amplification proved impossible. Re-precipitating the DNA with ethanol was carried out in an effort to remove some of the smaller sheared fragments (Sambrook *et al.* 1989) and BSA (Bovine serum albumen) was added (10 μg/ml) in an attempt to stabilise the polymerase enzyme (a technique commonly employed with restriction enzymes) and recently adopted by RAPD practitioners (e.g.Wilson *et al.* 1997) but both proved ineffectual. Ribosomal DNA primers, on the other hand, were

successful around 80 % of the time with the same DNA, presumably due to the high copy number of rDNA.

2.6.3. RAPD-PCR optimisation

Table 2.1 presents the optimised components for a 50 µl reaction volume. Thirty five cycles was chosen as standard with a two minute 95 °C denaturation prior to cycling followed by 95 °C 1 min, 36 °C 1 min then 72 °C 2 min. A final seven minute extension was carried out at 72 °C.

Table 2.1. Optimised PCR components for RAPD amplification

Reagent	Final Concentration	Amount used
Stoffel Buffer	1x	5 µl
$MgCl_2$	3.0mM	6 µl
dNTPs	100 µm	2.5 µl
Primer	200ng	2 µl
Stoffel *Taq*	0.5 Units	0.1µl
DNA	20ng	1µl stock DNA
SDW	1M	33.4 µl

The Stoffel buffer (Perkin Elmer, UK) comprised 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 0.001 % (w/v) gelatine. DNA template was present at a concentration of approximately 20-50 ng per reaction based on dilutions as described above. This typically corresponded to a 1:10 dilution of re-suspended genomic DNA in SDW.

The optimised magnesium concentration is higher than that typically used for standard *Taq* polymerase and this is in line with Stoffel's reported requirements (Perkin Elmer, UK). A high magnesium concentration is thought to enhance the stability of primer/template interactions (Welsh and McClelland 1991), which may explain Stoffel *Taq*'s reportedly superior ability to amplify smaller fragments. In actuality both Stoffel fragment *Taq* and "standard" AmpliTaq polymerase produced clear, repeatable results in early experiments (not shown). However, Stoffel *Taq* was chosen as the enzyme of choice since it has several reputed advantages over unmodified *Taq*, including a considerably longer (two-fold) half-life at high temperature (e.g. Bassam *et al.* 1992; Rafalski *et al.* 1996), improved amplification of smaller fragments (Erlich *et al.* 1991), as well as being less expensive.

Thirty-five cycles of amplification were found to produce RAPD profiles with a full complement of products, which had sharper and more easily distinguishable bands, with less smearing, than longer runs, and therefore the shorter number of cycles was adopted due both to the clarity of profiles and the time saved (Figure 2.3).

The optimised parameters were also tested without an initial denaturing step, as suggested by Beilawaski *et al.* (1995), who claimed that better quality RAPD profiles were obtained without this preliminary stage with vertebrate DNA. No major difference in profiles between samples denatured for 2 min at 95 ^{o}C prior to cycling and those not denatured was found (Figure 2.4). However, the 2 minute initial denaturation was continued to ensure the complete melting of all DNA bonds allowing the primer access to the entire genome. It would seem conceivable that

certain regions of DNA, such as those "unzipped" at an active replication fork, may be more accessible to primers, and a short denaturation may cause preferential annealing and subsequent amplification of these regions.

Of the two PCR machines tested, the Techne Genepro machine was considerably faster over 35 cycles than the Perkin Elmer TC-1, due to its superior cooling and heating ability. However, it was found that larger molecular weight bands were not always amplified by the faster machine (Figure 2.5) (perhaps due to the shorter length of time available for extension) and therefore the more consistent Perkin Elmer TC-1 was chosen despite the longer run time. These machine specific differences are a phenomenon well characterised (and criticised) in PCR studies (e.g. Penner *et al.* 1993), and reinforces that consistency in equipment and materials is vital in all PCR based assays.

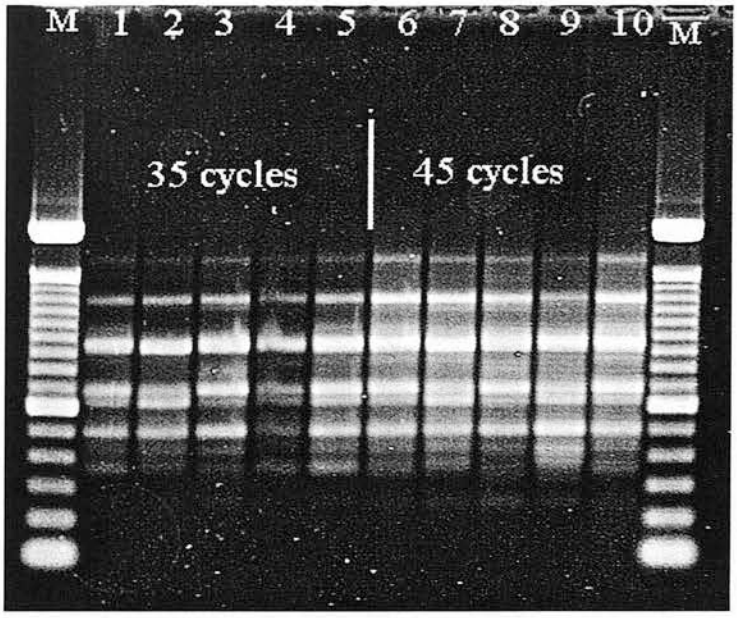


Figure 2.3. Effect of PCR cycle number on RAPD profiles.
Thirty five cycles or 45 cycles of RAPD amplification with five *Enoplognatha ovata* individuals, primer OP-AR19 (5'-CTGATCGCGG-3'). M= molecular size marker.

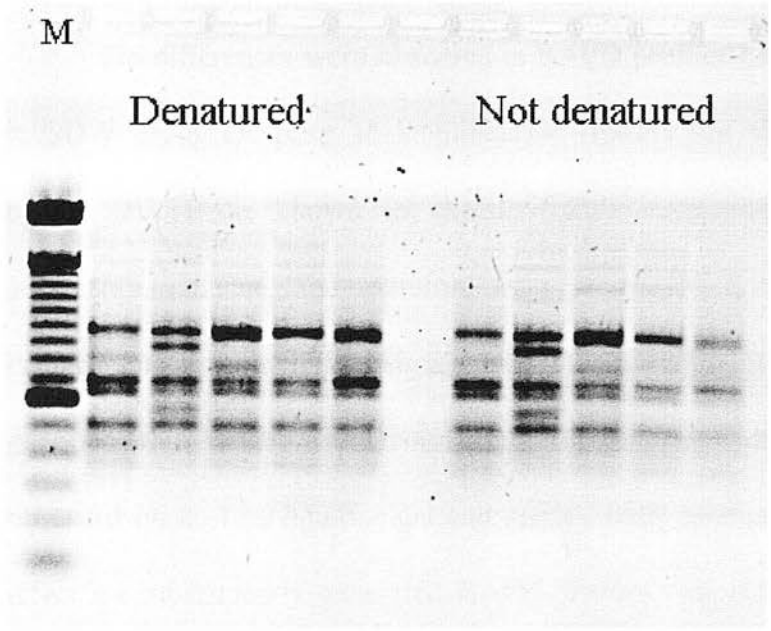


Figure 2.4. Effect of initial denaturation on RAPD profiles.
A RAPD reaction with either an initial denaturation of 2 minutes at 94 °C or no initial denaturation was carried out with five *Enoplognatha ovata* individuals, primer OP-AR19 (5'-CTGATCGCGG-3'). M= molecular size marker. Colour inverted image.

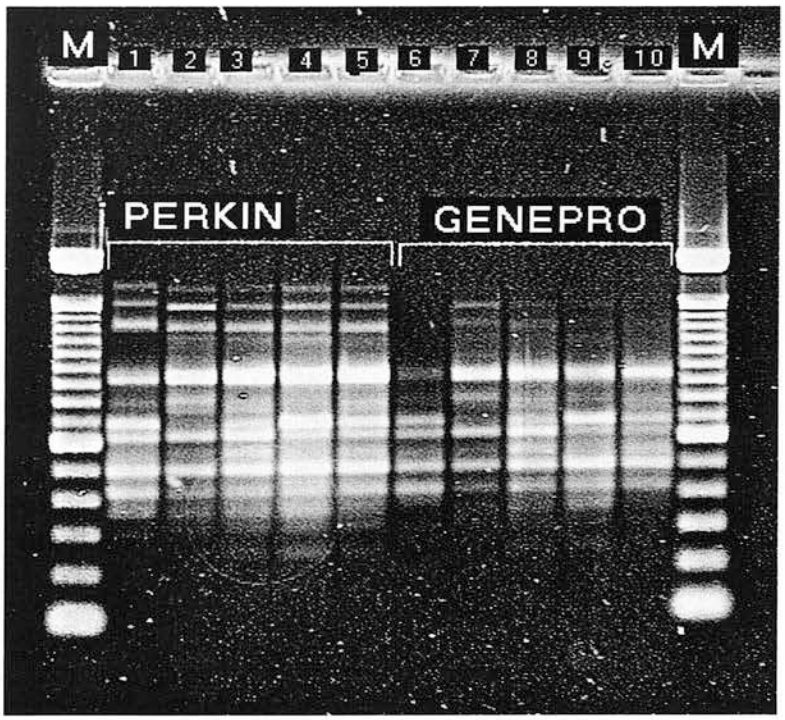


Figure 2.5. Effect of PCR machine on RAPD profiles.
Perkin Elmer GeneE vs Techne Genepro. Five *Enoplognatha ovata* individuals with primer OP-AR19 (5'-CTGATCGCGG-3'). M= molecular size marker.

2.6.3.1. DNA storage prior to RAPD amplification

No differences were observed in RAPD profiles generated from DNA stored in SDW or in TE prior to amplification (results not shown). However, EDTA, present in TE, is known to chelate (bind) magnesium, the co-factor for the polymerase enzyme, and therefore as a precaution against altering the level of magnesium present, SDW (Sigma, UK) was used for DNA storage. Treating the genomic DNA sample with RNase A had the desired effect of digesting the RNA (as observed on a 1 % agarose gel and stained with ethidium bromide). However, no effect on subsequently generated RAPD profiles was observed, indicating that the level of RNA present was not an inhibiting factor for amplification and its use was discontinued (a similar conclusion was reached by Zande and Bijlsma (1995)). Interestingly, the carapace typically contained far lower levels of RNA than the abdomen, and perhaps it was as a consequence of using the carapace for DNA extraction, that RNase was found unnecessary.

The RAPD profiles obtained from stock genomic DNA stored in SDW at 4 ^{o}C (then diluted to working concentration just prior to RAPD amplification) were found to produce consistent profiles up to a month after extraction (Figure 2.6). Diluted DNA i.e. at working concentration ready for use in PCR reactions, was found to produce more variable profiles over time, mainly in terms of intensity of the bands produced, rather than loss or gain of bands (Figure 2.7). Genomic DNA stored at -20 ^{o}C however, produced excellent reproducible profiles throughout an eight month period (results not shown). From the results obtained it appears prudent to make fresh dilutions from undiluted stock DNA held at 4 ^{o}C if the samples are to

be used presently, otherwise storage at -20 °C is advisable. Storing DNA at working PCR concentration for any length of time at 4 °C is not advisable.

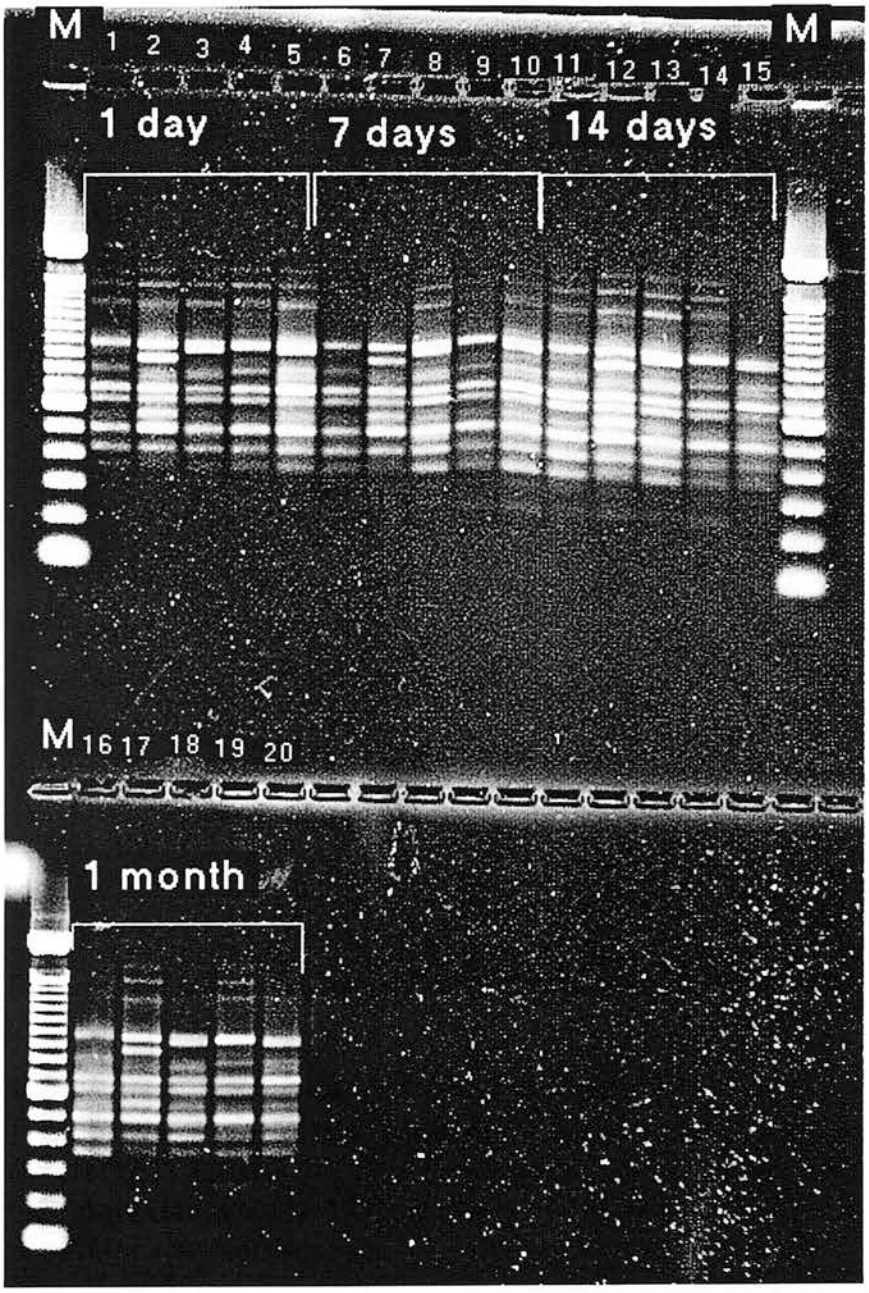


Figure 2.6. Reproducibility of RAPD markers.

Profiles from stock DNA extractions stored at 4°C with primer OP-AR19 (5'-CTGATCGCGG-3'). Five *Enoplognatha ovata* profiles after 1 day (lanes 1-5), 7 days (lanes 6-10), 14 days (lanes 11-15) and one month (lanes 16-20). M= molecular size marker.

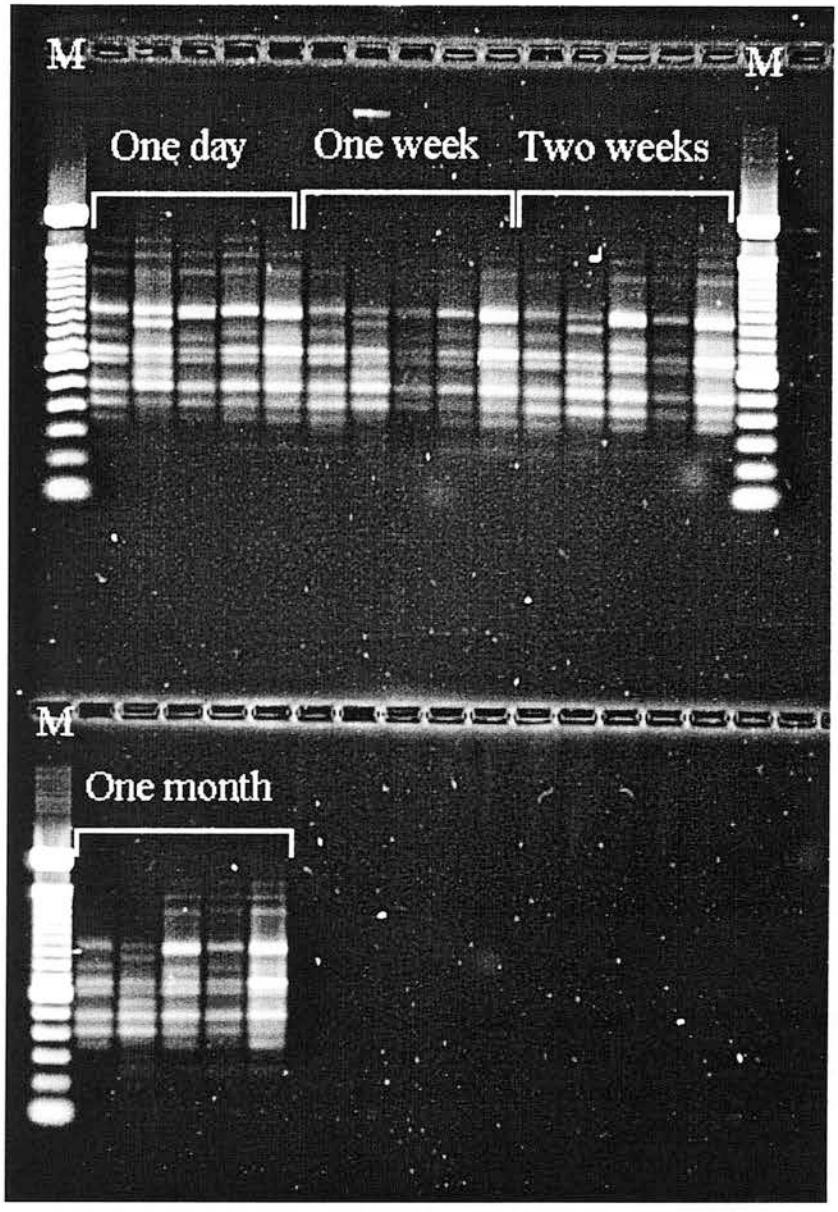


Figure 2.7. Reproducibility of RAPD markers.

Profiles from diluted DNA extractions stored at 4 ^{0}C, with primer OP-AR19 (5'-CTGATCGCGG-3'). Five *Enoplognatha ovata* profiles after one day, one week, two weeks and one month. M = molecular size marker.

2.6.4. Adaptability of technique

2.6.4.1. RAPD profiles from three spider families

The universal applicability of random primers is at the heart of the power of the RAPD technique, and the profiles presented in Figure 2.8 show that the optimised amplification conditions and ten-base primers successfully amplify DNA profiles from all three species of spider initially tested, emphasising that the technique is robust and transferable between spider families. These protocols have also been found to work with species of the genera *Meta*, *Stegodyphus*, and a number of linyphiid species. (results not shown).

Developing a RAPD-PCR protocol which does not require modifying components, such as the magnesium or primer concentrations, for each species studied is paramount for this technique, the simplicity of which is at the heart of its utility - and this has been achieved.

2.6.4.2. RAPD profiles from spiderlings

Although not applied in this study, these protocols enable RAPD profiles to be generated from individual second instar spiderlings with all primers tested (See Figure 2.9 for profile generated with primer AR19). This is not unexpected given the power of PCR to amplify from individual mites, micro-Hymenoptera and other small invertebrates (e.g. Fenton *et al.* 1997; Landry *et al.* 1993). Without careful mating studies it is impossible to confirm the Mendelian inheritance of RAPD markers in spiders (as outlined by Williams *et al.* (1990), Dawson *et al.* (1993) and Stott *et al.*

(1997) in other organisms) but given the appropriate conditions this would seem a practical possibility.

These results do indicate though, that with adequate screening, RAPD-PCR markers could be generated which would allow the identification, and hence inclusion, of immature and sub-adult spiders (i.e. prior to genital formation) in spider density studies, which often contain large numbers of morphologically unidentifiable immature spiders.

Figure 2.8. RAPD profiles produced with three primers from three spider families.
OP-B03 (5'-CATCCCCCTG-3'), OP-AF15 (5'CACGAACCTC-3') and OP-AR19 (5'-CTGATCGCGG-3') (chosen at random from those available in the laboratory) with five individuals from (a) *Lepthyphantes tenuis* (b) *Clubiona reclusa* and (c) *Enoplognatha ovata*. M= molecular size marker.

(a)

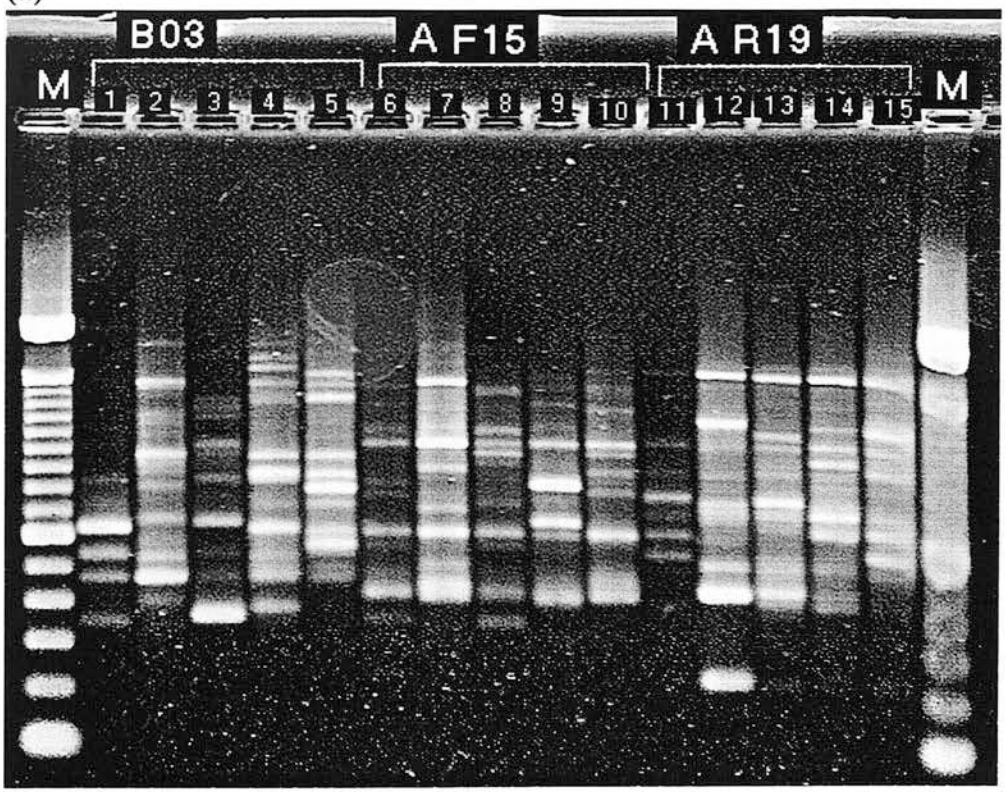
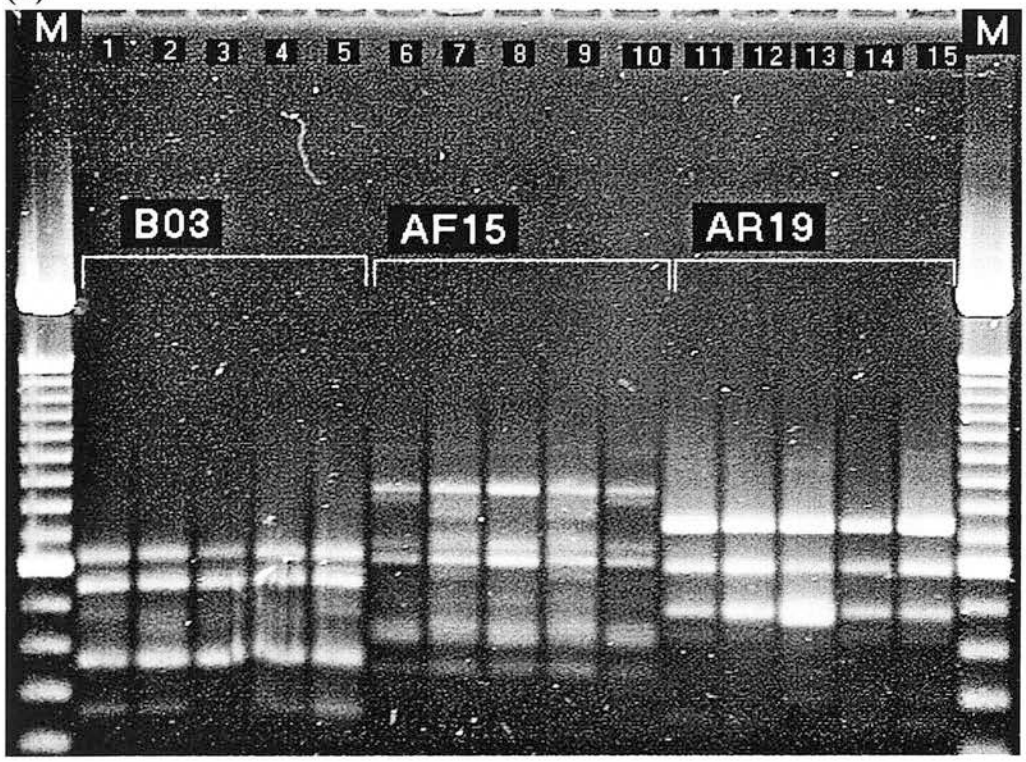
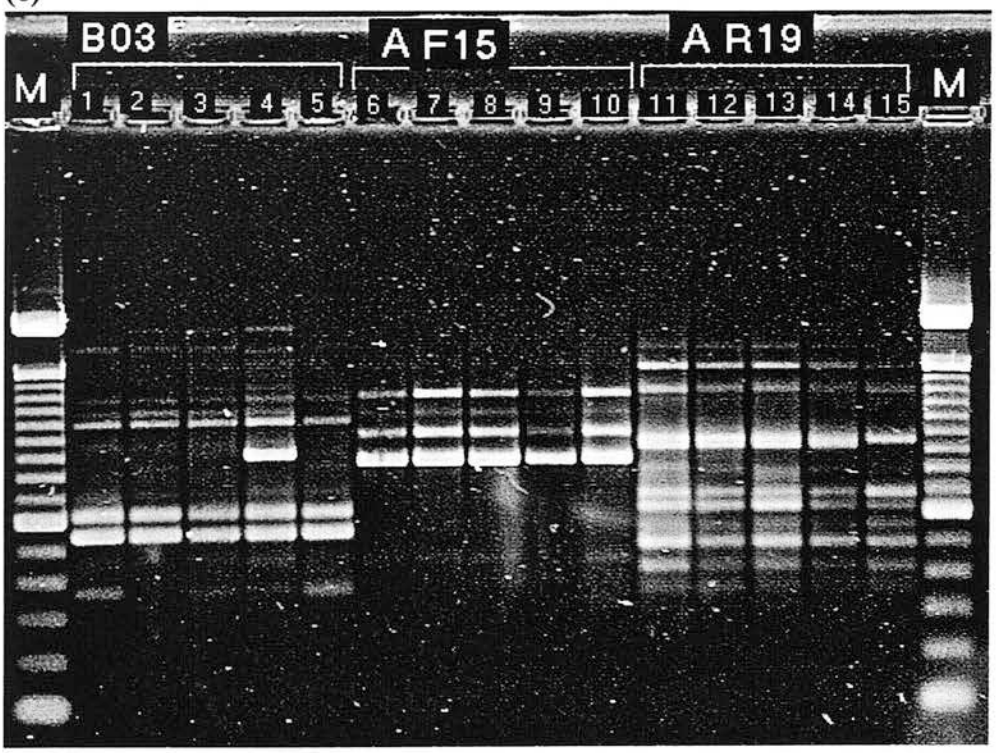


Figure 2.8. continued

(b)



(c)



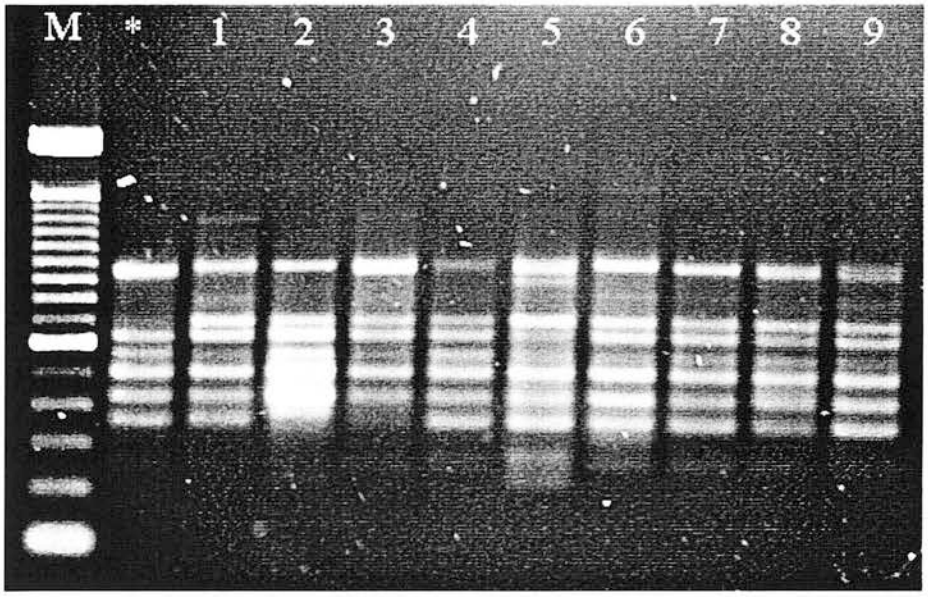


Figure 2.9. RAPD profile generated from a mother and spiderlings. ***Lepthyphantes tenuis*** **(*) and nine second instar offspring with primer OP-AR19 (5'-CTGATCGCGG-3'). M= molecular size marker.**

2.6.5. Homology of RAPD bands

The homology of RAPD bands is often at the heart of criticism of the technique. Is the resolution of a 1.4 % agarose gel, and the human eye, sufficient to assess the similarity of bands? Indeed, even if the bands *are* exactly the same length of sequence, what is the guarantee of actual nucleotide sequence similarity? By employing restriction enzyme analysis on re-amplified bands it can be demonstrated that at least some of the products do, in fact, show a degree of sequence homology. The results raise many interesting points. The three enzymes employed (*Eco*RI, *Dde*I and *Hae*III) all produced the same restriction patterns (*Eco*RI did not restrict) with the fragment from the *E.ovata* from Invergowrie, Dundee and Blackford Hill, Edinburgh, giving good indication of sequence homology (Figure 2.10 presents the *Dde*I digest).

However, interestingly, it becomes apparent that there are in fact multiple amplification products present - a 1000 bp fragment is still present despite digestion - in what would be scored as only a single band. This is a reflection of the limitations of agarose gel to separate fragments which are within up to approximately a 50 bp range (electrphoresing the sample on a polyacrylamide gel may have resolved the bands). Nevertheless, the main outcome of this small scale experiment is that in all probability the fragments which are being scored are homologous loci (or at least contain multiple homologous loci), and therefore a valid comparison is still being made.

A less cumbersome approach to assessing the homology of RAPD fragments, which was only conceived following the completion of practical work, would be to simply add restriction enzyme to the whole RAPD post-amplification mix (preferably using a primer which amplifies only a few bands) and compare restriction profiles between samples. This approach would again allow the assessment of homology of bands, but without the effort of gel purifying and concentrating a single fragment.

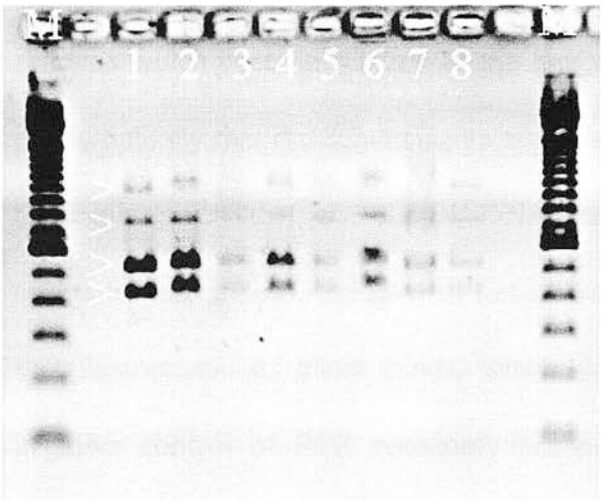


Figure 2.10. Homology of RAPD bands.
***Dde*I digest of 1000 bp re-amplified RAPD band. *Enoplognatha ovata* from Invergowrie (lanes 1-4) and Edinburgh (lanes 5-8). The white arrows indicate the position of the four products. M= molecular size marker. Colour inverted image.**

2.6.6. Screening for potential contaminants

2.6.6.1. Abdominal vs carapace generated profiles

A RAPD profile generated with three primers and 12 *E.ovata* individuals did not generate any bands present in the abdomen which did not appear in the carapace (results not shown). However, the occasional presence of larval parasites, the presence of the digestive mass, high levels of RNA, and the pigmentation of the opithosoma (high levels of pigmentation are thought to interfere with PCR) justified the removal of the abdomen.

2.6.6.2. Microbial contamination

RAPD amplification of a number of concentrations of the fungal contaminants produced weak amplification in the case of one primer, and no

amplification with the other. This was in contrast to strong profiles generated with *L.tenuis* which bore no relation to the fungal profiles (results not shown). It seems highly unlikely that the contaminants are contributing to the spider profiles obtained, especially given that the fungal DNA template was at an unrealistically high concentration. Indeed, the level of fungal contamination may contribute, at most, to the phenomenon of ghost bands, which are occasionally found to appear in the negative control of PCR reactions. These are often found to disappear on the addition of template DNA, indicating they have no input to the overall profile. As a precaution however, if any specimens appeared parasitised or fungally infected at the specimen identification phase, they were excluded from further analysis.

2.6.7. Amplification with universal primers

2.6.7.1. Mitochondrial DNA amplification

Despite numerous modifications, including titration of magnesium and DNA concentrations, and attempting a number of different annealing temperatures, no product of the expected range (1400 bp) could be amplified, although two much smaller products (approximately 200 and 350 bp) were often recorded (Figure 2.11).

As an effort to aid amplification, the primers were heated to 100 ^{o}C to separate any PCR-function-inhibitory primer-dimers (primer-primer annealing) which may have occurred over several freeze-thaw cycles, then flash frozen in liquid nitrogen - a technique known as "primer reviving". However the same results were consistently obtained. The small amplified products may have been a spider mitochondrial fragment, a primer-dimer (small PCR products generated by primers

binding to each other and/or incomplete fragments), or even a RAPD type amplification occurring with one of the specific primers. With sufficient resources the product could have been cloned and sequenced to reveal its origin, but it was beyond the scope of the project. Furthermore, as success was being obtained with the conserved ribosomal primers, it was decided to concentrate on that avenue of investigation.

2.6.7.2. Ribosomal DNA amplification

The PCR conditions presented in Table 2.2 were highly successful at amplifying spider rDNA. Please see Chapter Five for full details, including primer sequences.

The following cycling conditions were used on a Techne GeneE PCR machine. A two stage amplification cycle was employed - 25 cycles of 1 min 94 ^{o}C, 1 min at 58 ^{o}C and 2 min at 72 ^{o}C, followed by 15 cycles of 1 min at 94 ^{o}C, 3 min at 58 ^{o}C and 3 min at 72 ^{o}C, all performed at maximum ramp rate. A final primer elongation stage of 5 minutes at 72 ^{o}C to complete all fragments was then carried out. The second round of thermal cycling allowed a greater length of time for primer annealing and subsequent elongation, in what would be a primer and dNTP depleted master mix. A layer of mineral oil was not required (cf. the RAPD-PCR reaction) as this particular PCR machine was equipped with a heated lid, which prevents evaporation of the sample by maintaining a constant temperature across the whole tube.

Table 2.2. Optimised PCR components for rDNA amplification

Reagent	Final Concentration	Amount used
Buffer	1x	5 µl
$MgCl_2$	1.5mM	3 µl
dNTPs	100 µm	2.5 µl
Primer	100ng	1 µl
Taq polymerase	0.5 Units	0.1µl
DNA	20 ng	1µl
SDW	1M	37.4 µl

Approximately 20 ng (1 µl) of high molecular weight genomic template DNA extracted as described previously was used in each reaction. The PCR buffer and *Taq* polymerase were supplied by Flowgen, UK. To check if a successful amplification had occurred, 8 µl of reaction mix was added to 3 µl loading buffer and co-electrophoresed with a molecular marker on a 1.4 % agarose gel. The gel was stained with ethidium bromide, UV illuminated and a visual record of the gel made (see Section 3.3.1). Figure 2.12 shows an example of the amplification of the internal transcribed spacer (ITS1) region of *L.tenuis* and *E.ovata*.

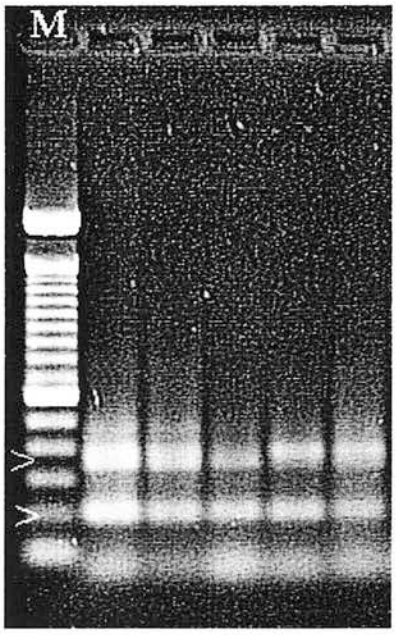


Figure 2.11. PCR of mitochondrial fragments from five *Enoplognatha ovata*. The white arrows indicate the 200 and 350 bp products consistently amplified. M= molecular size marker.

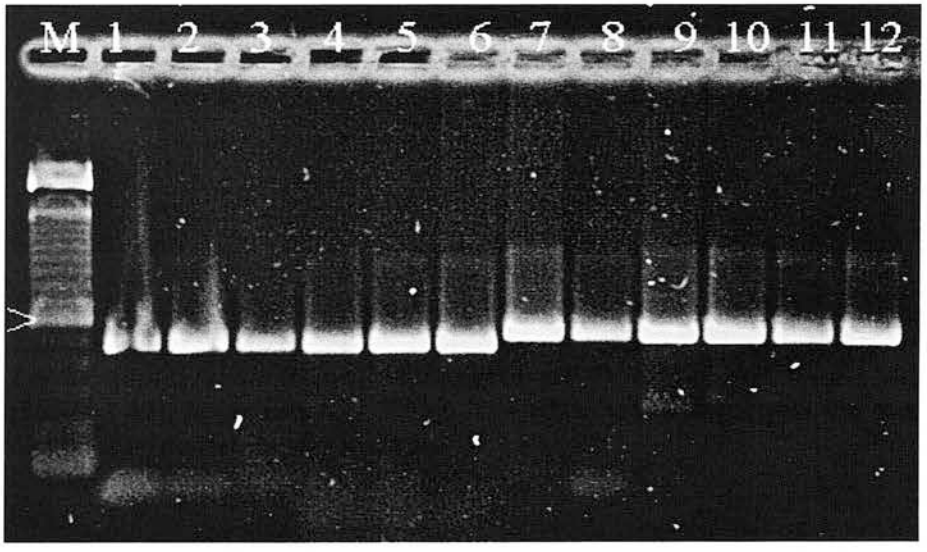


Figure 2.12. PCR amplification of the ITS1 region of spider rDNA.
Example of amplification with primers C and B of the ITS1 region of six *Lepthyphantes tenuis* (Lanes 1-6), approximately 450bp; and six *Enoplognatha ovata* (Lanes 7-12), approximately 500bp. The white arrow highlights the marker band at 600 bp. M= molecular size marker.

2.7. Summary

These preliminary studies have developed protocols for effective methods of sample preservation, DNA extraction and storage, and PCR amplification of spider DNA. In addition to the optimisation of RAPD-PCR protocols, the potential problems of homology, contamination and repeatability have also been addressed. The technique has proved powerful and repeatable with all spider samples tested.

The insect-specific mitochondrial primers proved unsuccessful in amplifying spider DNA. This element of the study was not pursued further. In contrast, the ribosomal DNA primers proved highly successful at amplifying the spider homologue. Only minor changes to the PCR protocol of Fenton *et al.* (1997) were required.

In Chapter Four, the RAPD technique is examined in greater detail and results of spider work are presented and analysed, and in Chapter Five the rDNA study is expanded to include PCR-RFLP analysis, cloning, and sequencing.

3. GENERAL MATERIALS AND METHODS

3.1. Sampling

In regards to sampling, every study must be tailored to logistical limits relative to the equipment, materials and personnel available, with some assumptions made at the outset to facilitate the planning and execution of field work. In this study, population variation was investigated at two levels; at the local scale (< 100 km) via RAPD analysis, and across a broader geographic scale via rDNA analysis.

For the rDNA section of this study, *E.ovata* were sampled from sites within Scotland (Elgin, Invergowrie and Edinburgh), since it was reasonable to hypothesise that lower levels of gene flow would be occurring between populations of this more sedentary species, which may lead to genetic differentiation detectable over this relatively short geographic range. The site Blackford Hill in Edinburgh, in particular, is located centrally in the city and is therefore unlikely to receive many new immigrants.

The *L.tenuis* for rDNA analysis, in contrast, were sampled from sites geographically further apart - from Scotland, England and New Zealand, reflecting the hypothesis that genetic differentiation, if present, would only be detectable over a larger scale (due to the ballooning ability and hence homogenising gene flow of this species). If these distant populations were differentiated, samples from intermediate distances could then be screened until a cut off point, indicative of effective population size, was identified.

3.2. Spider collection and identification

3.2.1. Collection

Scottish *L.tenuis* samples were collected in July/August 1996 from winter wheat (cultivars unknown) at seven sites between Edinburgh and Dunbar in East Lothian, and from three East Lothian sites in July 1997 - Boghall, Phantassie and Spotte (see Figure 3.1) using a D-vac insect suction sampler (Dietrich 1961). Sampling was either carried out at field margins or by randomly placing the D-vac nozzle into the crop either side of the tractor tram lines. Hand sampling was also undertaken but proved an unproductive means of collecting sufficient numbers of individuals. *L. tenuis* were also obtained from Littlehampton, England and from New Zealand in 1996.

Although not quantified accurately during sampling, the highest abundance of linyphiid spiders corresponded to areas of high weed coverage within the field, in accordance with the findings of Topping and Sunderland (1994a). This is thought to be due to the relatively moist conditions present in weedy patches which create a favourable microhabitat for invertebrates. This finding allowed the D-vac sampling effort to be concentrated on areas within the crop which would yield the highest number of spiders.

The *E.ovata* samples in 1996 were collected from nine roadside sites bordering cereal fields in East Lothian, one site at Invergowrie, Dundee, and one sight near Elgin, in late July/August by hand collection from Bramble bushes, which provide a suitably habitat for gravid females. These Theridiion spiders are easily distinguished by size, shape and colouration (Figure 3.2a,b). In 1997, *E.ovata* were

again collected from Invergowrie - to allow a temporal comparison of genetic variability; from Blackford Hill, Edinburgh, and from Spotte and Phantassie, East Lothian. Table 3.1 presents the ratios of each of the three abdominal colour morphs at the four 1997 sites in and from Invergowrie in 1996.

Table 3.1. Abdominal colour morph frequency of *Enoplognatha ovata* 1997 samples

	Yellow	Striped	Red
Invergowrie (1996)	52	22	1
Invergowrie	40	7	1
Blackford Hill	35	12	1
Spotte	30	15	3
Phantassie	18	10	0

The yellow > striped > red morph frequency is the situation which occurs across all *E.ovata* populations world-wide (Oxford and Shaw 1986). The genetic basis for the colour polymorphisms has been examined in detail by Oxford (1983) and will not be addressed in this study.

Unfortunately, it must be noted that whilst spiders were collected and identified in 1996, and a proportion used in the preliminary experiments, they are not included in the main body of the work as uncontrollable circumstances (freezer failure and the subsequent degradation of DNA) precluded their use.

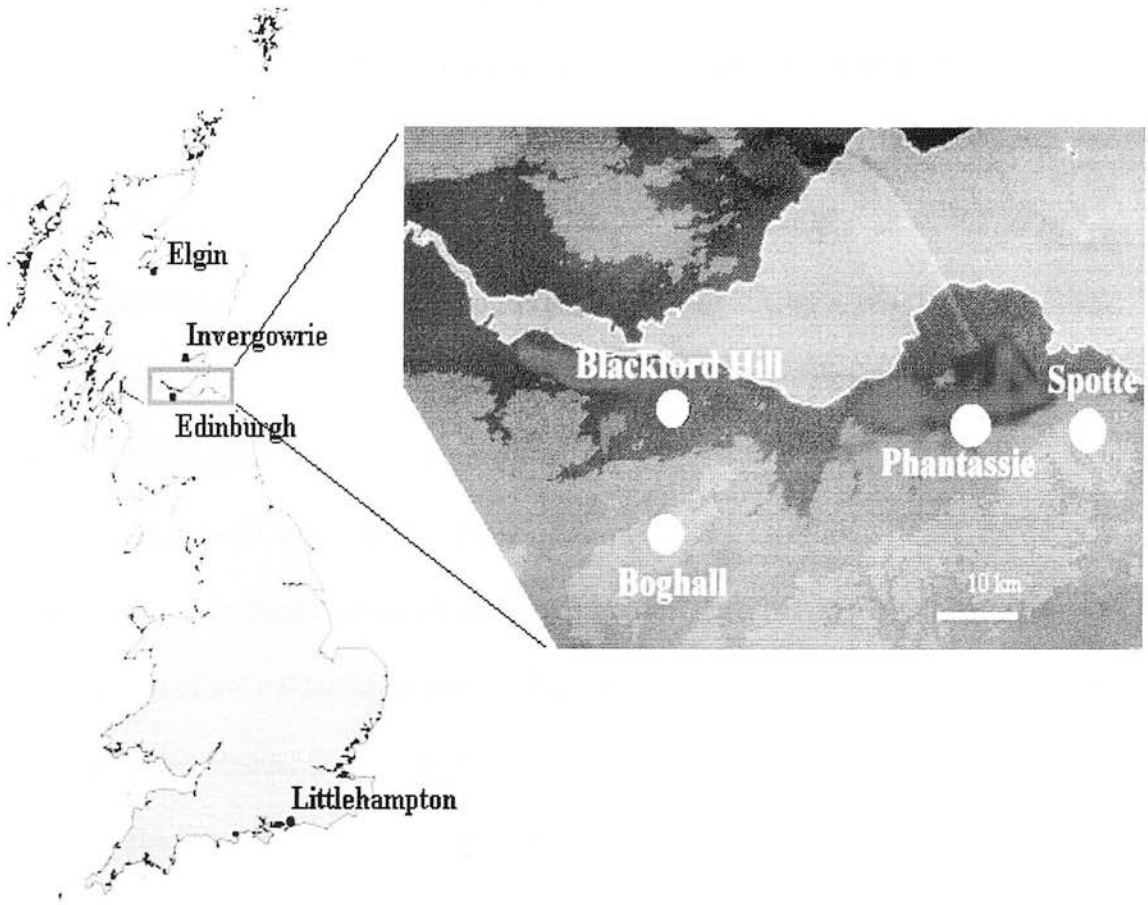


Figure 3.1. Map of British spider sampling sites.
The highlighted box shows East Lothian in greater detail. These populations were sampled in 1997. *Lepthyphantes tenuis* from Littlehampton and *Enplognatha ovata* from Elgin were collected in 1996.

3.2.2. Spider identification

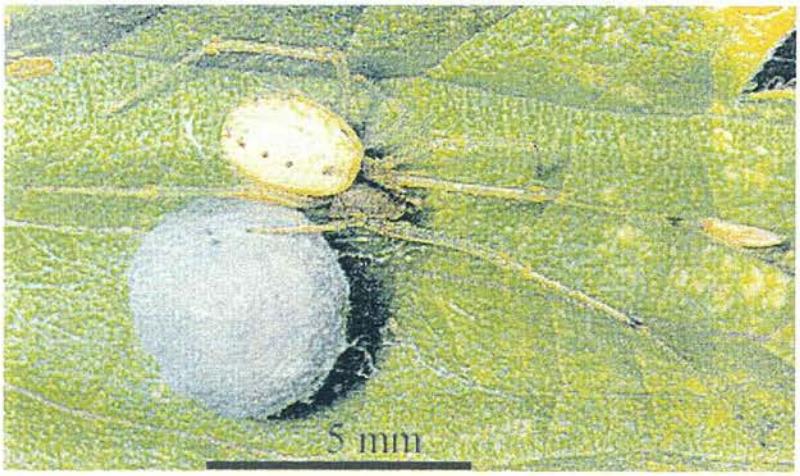
The D-vac samples were returned to the laboratory where Linyphiid spiders were separated by eye from the other spiders and organisms present (by shape and size) and pootered into Petri dishes lined with moist filter paper (small spiders are particularly prone to desiccation). These were held overnight at 4 ^{o}C prior to speciation. Spiders are poikilothermic organisms (maintaining a widely variant body temperature, usually close to the variation in ambient temperature) and are easier to handle when subjected to low temperatures.

Members of the *Lepthyphantes* genus were distinguished initially by general size, shape, abdominal markings and colouration (Figure 3.3a), then speciated by examination of genital structure under a light microscope (x 80) using Roberts (1987) as a reference manual (Figure 3.3b). Individual spiders were placed in a small Petri dish and the lid lifted to allow a pipe from a CO_2 cylinder to deliver a short burst of gas (approximately 5 seconds). The narcotised spider was then placed in the bottom of a watch glass, gently flipped over and the epigyna examined under the microscope using a swan-necked optic light box as a directable light source.

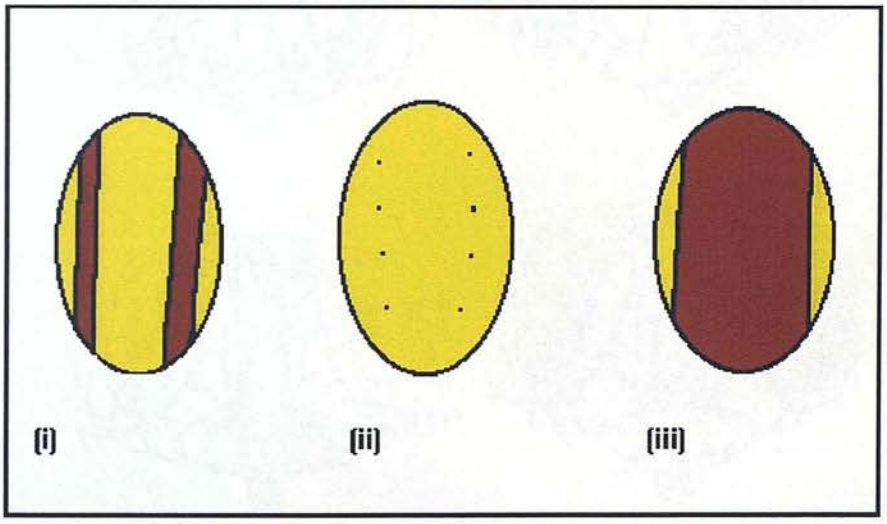
L.tenuis was by far the most common *Lepthyphantes* spp. found (> 95 %), followed by fewer individuals of *L.cristatus* (Menge) and *L.zimmermanni* (Bertkau). Large numbers of *Erigonae* spp. were also found at all sites. These are ground dwelling spiders which are picked up easily by the D-vac in addition to spiders in the vegetation. In addition, large numbers of immature spiders were collected in the D-vac samples. These often cannot be identified beyond family due to the lack of mature sexual organs for identification.

A number of adult female *L.tenuis* spiders were returned to paper-lined Petri dishes and maintained at 18 ^{o}C in anticipation of the production of an egg sac. The paper required moistening approximately every 48 hours to prevent the death of the spider due to desiccation. Aphids and other small invertebrates, collected co-incidentally whilst D-vac sampling, were brushed into the Petri dishes at regular intervals to supply a food source. Second instar spiderlings (which emerge directly from the egg sac) were frozen on the day of emergence at -80 ^{o}C, with their mother, to facilitate future study.

Only female spiders of both species were used in the course of this study, due both to their relative ease of identification and because, certainly in the case of *L.tenuis*, females exert greater predation pressure on pests and are more dispersive than males (Topping and Sunderland 1998).

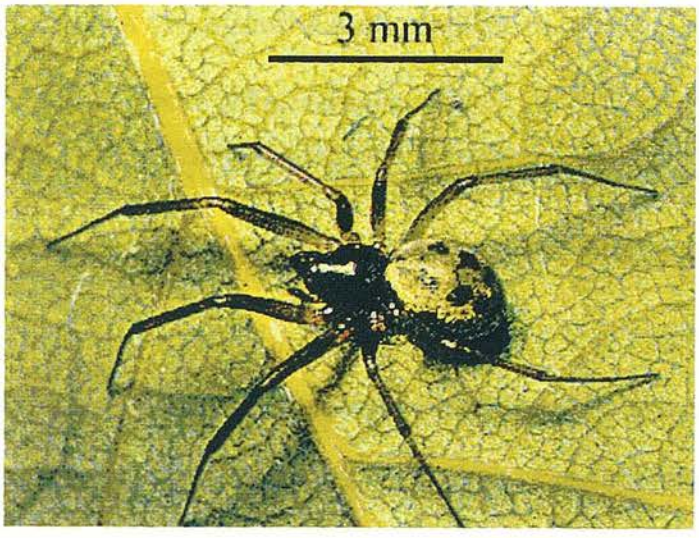


(a)

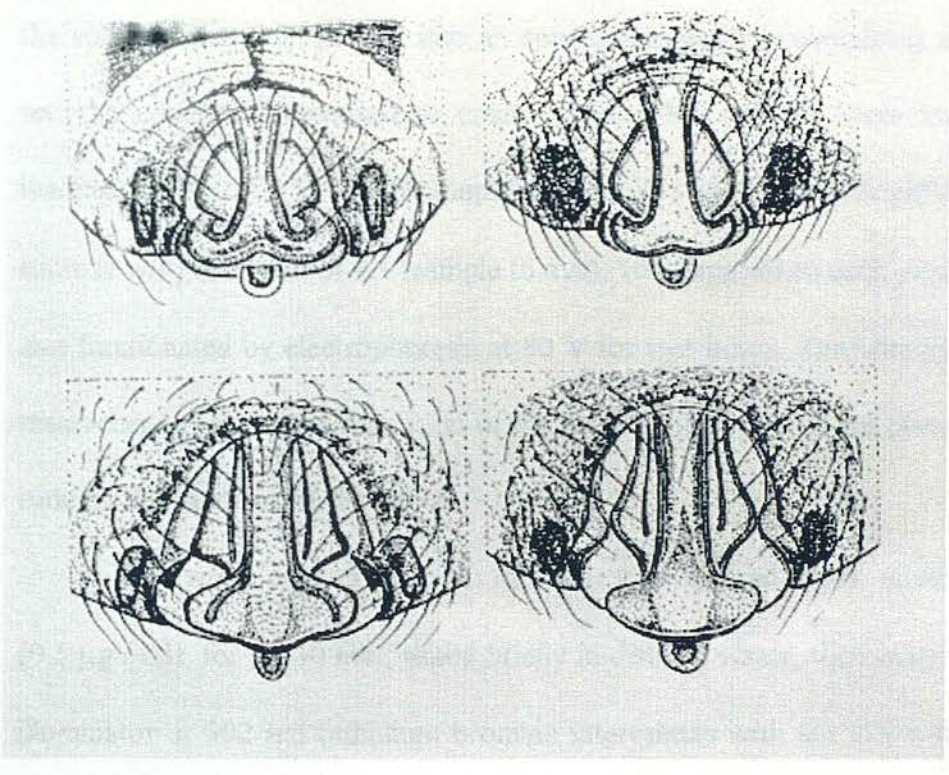


(b)

Figure 3.2. ***Enoplognatha ovata.***
(a) ***Enoplognatha ovata*** **female with egg sac (From Roberts 1987).**
(b) Stylised diagram of ***Enoplognatha ovata*** **abdominal colour polymorphisms; (i)** ***E.ovata*** **var. redimita (ii)** ***E.ovata*** **var. lineata (iii)** ***E.ovata*** **var. ovata. Due to the bright colours the redimita morph has gained the nick-name of the Candy-stripe spider in North America.**



(a)



(b)

Figure 3.3. *Lepthyphantes tenuis.*
(a) Female *Lepthyphantes tenuis* with typical abdominal markings (black spots meeting in centre).
(b) Diagrams of *Lepthyphantes* spp. epigynal structure (x80). Two examples of typical epigynal structure are shown for *L.tenuis* (top) and *L.zimmermanni* (bottom) (From Roberts 1987). *L.tenuis* has a characteristic "anchor" shaped structure.

3.3. DNA visualisation and quantification

3.3.1. Agarose gel electrophoresis

Amplified DNA products were visualised on either a 1.4 % TAE (40 mM Tris, 20 mM acetic acid, 1mM EDTA) or TBE (89 mM Tris-HCl, 89 mM boric acid, 5 mM EDTA) agarose gel prepared following standard protocols (Sambrook *et al.* 1989). Genomic extractions were run on 1 % agarose gels, due to the considerably larger molecular weight of the DNA. Briefly, the required amount of agarose (Biorad, UK) was added to the buffer in a conical flask and microwaved until the agarose dissolved completely and the solution became clear. When cooled to 60 ^{o}C, the solution was then poured into an appropriate gel tray containing a comb. Once set, the comb was removed to create wells. DNA samples were mixed with gel loading buffer (0.05 % w/v bromophenol blue, 0.1 % w/v EDTA pH 8.0 and 40 % sucrose w/v) at a ratio of 4:1 (sample to dye), 10 µl applied to each precast well, then size fractionated by electrophoresis at 80 V for two hours. The blue dye enables the researcher to follow the migration of the samples through the gel and prevent over-running the samples off the gel.

Following electrophoresis, the gel was stained with ethidium bromide (0.5 µg / ml), for 20-30 min, rinsed briefly in distilled water, then examined on a UV illuminator at 302 nm (ethidium bromide intercalates with the DNA fragments and fluoresces under UV illumination). The results were captured on screen and saved to hard disc using the IS500 digital image analysis system (Flowgen, UK). This system allows a "negative" inverted image to be printed, and these were used for analysis if it

made the band patterns more readable. This approach has been applied by many authors (e.g. DeBarro *et al.* 1995).

TAE is reported as possessing superior buffering capacity to TBE, allowing better resolution of larger fragments, but poorer resolution of smaller fragments (Dowling *et al.* 1996), but no discernible differences were noted in this study. Varying the thickness (i.e. depth) of the gels and the percentage of the gels (between 1-2 %), had little or no effect on the clarity of RAPD profiles. Ensuring the gels were firmly set by placing them at 4 ^{o}C for 30 min prior to electrophoresis was found to improve band sharpness, presumably by "tightening" the agarose matrix through which the DNA fragments run and are size separated. Using polyacrylamide gels to separate RAPD fragments, as expected, produced a greater number of scorable fragments (results not shown), but the high cost of the gels precluded their routine use.

As a possible avenue to gaining intermediate resolution between agarose and polyacrylamide, Metaphor XL (Flowgen, UK) gels were tested. This is a high cost agarose which is cast at between 3 and 4 % and is reputed to produce a superior resolution to agarose. However, the gels were found to be difficult to handle and had a preparation time considerably longer than that of agarose, and again the benefits of conventional agarose outweighed the reduced level of separation.

3.3.2. Polyacrylamide gel electrophoresis

Both pre-cast (Pharmacia, UK) and self-cast gels were employed in the course of the study. The self-cast gel was prepared in three stages - the plug gel (to

prevent leakage from the bottom of the gel rig), the resolving gel (which separates the fragments) and the stacking gel (which aligns the samples prior to entering the resolving gel). The resolving gel was either 10 % or 16 % acrylamide w/v pH 8.7, and the stacking gel (5 %) pH 6.75. Tris/glycine was used as the buffer system. These vertical gels were typically run overnight at 70 V. The pre-cast gels were 12.5 % polyacrylamide and were run at the manufacturer's recommended 600 V for approximately 1.5 hours on a horizontal gel rig (Pharmacia LKB 117 Multiphore II). Due to the high voltage of this system the plate of the rig was connected to a circulating water unit set at 15 ^{o}C to cool the plate during electrophoresis. To ensure good electrical conductivity, approximately 1 ml of 1 % Triton X was applied to the gel plate prior to lowering the gel into position. Gels were stained with ethidium bromide as with agarose, UV illuminated, and an image captured.

3.3.3. DNA quantification

Spectrophotometer readings of DNA extractions were taken on a DU-65 spectrophotometer (Sambrook *et al.* 1989). DNA concentration is considered to be a particularly important factor in the reproducibility of RAPD results e.g. McClelland and Welsh (1994), and consistency of template quality and quantity is therefore essential. However, spectrophotometer readings were found to be unrepeatable, probably due to the level of proteins complexed with the DNA (Bruford *et al.* 1992) which cause the readings to "wobble". Concentrations were therefore calculated by comparing, by eye, the intensity of EtBr stained genomic extracts with dilutions of a high molecular weight marker (λ/HindIII digest) whilst under UV illumination

(Sambrook *et al.* 1989). This enabled dilutions of DNA to be made for PCR reactions which were a good approximation to each other. Interestingly, Zang and Hewitt (1998b) compared the effectiveness of DNA quantification via spectrophotometry and gel electrophoresis, and concur with this study, stating that it is generally better to estimate DNA quality via the gel electrophoresis method.

4. RAPD ANALYSIS OF SPIDER POPULATIONS

4.1. Introduction

RAPD-PCR can perhaps be best described as the Jeckyll and Hyde of molecular techniques - adopted wholeheartedly by many researchers due to its approachability in terms of implementation, yet possessing an often criticised "darker" side in terms of intellectual and theoretical limitations. This chapter critically examines both the benefits and potential pitfalls of the technique.

The theoretical mechanics of the RAPD-PCR reaction are as follows. At relatively low temperatures (35-40 ^{o}C), any short random primer (typically a 10 base primer with 50-60 % GC content) is likely to find, simply by chance, complementary sequences in a genomic template DNA to which it can anneal (i.e. within the many millions of bases constituting a genome there is a good probability of the complement of any 10 base sequence occurring). If the frequency of these sequences in the template is relatively high, there will be a further chance that pairs of sequences will lie close to one another. If the annealed primers are arranged in an opposite orientation on both DNA strands, such that the 3' ends of the primers point to each other (generally within < 2000 bp), the PCR reaction will amplify the fragment between the primers. When this occurs throughout the genome a set of fragments is produced which can vary in their presence or absence between different individuals, isolates, subspecies, and so on. (Figure 4.1). Polymorphisms between different DNA samples may be detected under a number of circumstances. The primary reason is if the primer is unable bind to a site in one sample due to non-complementarity, but can bind successfully in another. One base has been found to make the difference between

priming and not priming - indicating the sensitivity of the method (Williams *et al.* 1990). The detection of polymorphic markers may also result from deletions within a priming site, insertions that separate priming sites by too great a distance to support amplification, or insertions that change the length of the fragment without affecting amplification.

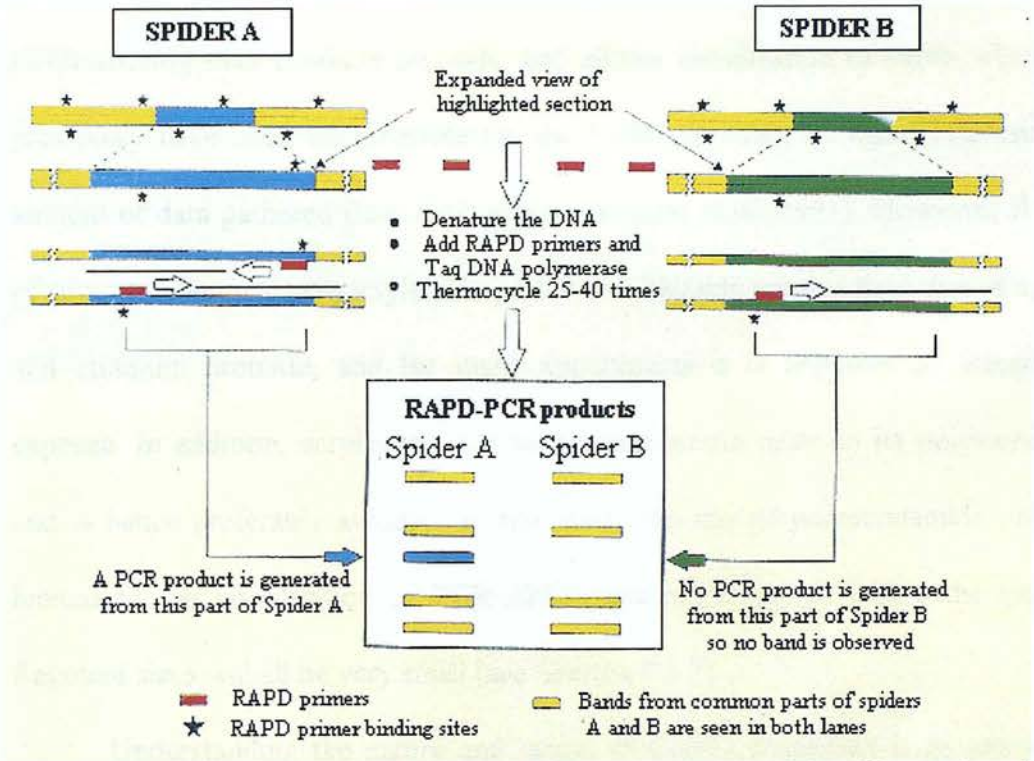


Figure 4.1. Representation of the RAPD-PCR reaction with spiders, A and B.

Electrophoresing the RAPD-PCR products through a gel matrix to separate the amplified fragments, and applying a DNA stain, produces a banding pattern typically involving up to a dozen bands (depending on the particular primer used and the complexity of the target genome). By sequentially screening the target genome with a set of random primers a potentially limitless number of loci can be generated for analysis. Typically, agarose gels (ranging from 0.8 to 3 %) stained with ethidium

bromide are used to separate and visualise the amplified fragments, but increasingly silver staining of polyacrylamide gels (ranging from 5-12 %) has become common e.g. Naish *et al.* (1995). The decreased matrix pore size of the polyacrylamide gels enables the resolution of very small fragments of DNA (< 0.1 kb) and hence often produces more data per primer (see He *et al.* 1994 for an appraisal of different gel matrices). Silver staining has been demonstrated to be a more sensitive method of DNA staining than ethidium bromide, and allows visualisation of bands which may previously have been undetectable (in the 1-100 pg range) - again increasing the amount of data gathered from each primer (Bassam *et al.* 1991). However, the cost of silver staining and polyacrylamide gels is considerably greater than that of agarose and ethidium bromide, and for many applications it is arguably an unnecessary expense. In addition, acrylamide is a potent neurotoxin prior to its polymerisation, and is hence preferably avoided. In this study the use of polyacrylamide gels was limited to the visualisation of PCR-RFLP restriction digests where the expected fragment sizes will all be very small (see Section 5.2.3).

Understanding the nature and origin of RAPD fragments is paramount for their use as genetic markers, and has come under some considerable scrutiny. Cloning and probing experiments with labelled RAPD products from *Glycine soja* and *G.max* (Williams *et al.* 1990) exhibited that both repetitive, (similar to those used in mini and microsatellite fingerprinting) and non-repetitive regions of DNA are amplified during RAPD-PCR. This experiment indicated that RAPD markers

are amplified from a range of DNA classes and not a predominant sub-set, which means that results of a RAPD assay should be unbiased. Importantly, Williams *et al.* (1990) and Dawson *et al.* (1993), with *Glycine* spp. and *Hordeum* spp. respectively, and more recently Stott *et al.* (1997), with the lake trout *Salvelinus namaycush*, have shown via breeding studies that RAPD amplified fragments are inherited in a bi-parental Mendelian manner, and are therefore valid as molecular markers for both qualitative and quantitative traits.

The power of the RAPD technique lies quite simply in its ability to identify polymorphisms in organisms where morphological and other biochemical methods prove problematic in highlighting variation. For example, as broached in Chapter One, many of the Homoptera have been found to possess very low levels of allozyme variability, whereas RAPD analysis detected variability even at the level of colour morphs of the same aphid biotype (Black *et al.* 1992). One of the reasons RAPD-PCR is so powerful is that theoretically the whole genome, including both coding and non-coding regions, is assayed simultaneously, in contrast to protein or site-directed PCR reactions which are only examining a restricted portion of the total DNA of an organism. This screening ability of a RAPD reaction immediately increases the probability of detecting polymorphisms. In addition, the mechanics of the polymerase chain reaction means that only small quantities of initial template DNA are required, enabling studies on small invertebrates to be carried out with relative ease (e.g. Roosien *et al.* 1993; Landry *et al.* 1993).

Unsurprisingly, due to its ease of application and "universality" across all genomes, an explosion of papers in the literature has occurred over the last six years

as applications for RAPD assays have become apparent and exploited (for an early review see Hadrys *et al.* 1992), and a literature search in almost any scientific database will quickly cite many dozens of references utilising the technique. A brief overview of some pertinent papers follows, with an emphasis on invertebrate RAPD studies.

4.1.1. Applications of the RAPD technique

One immediate application of RAPD markers was in the arena of identification. Reproducible genetic markers used for identification are important particularly with cryptic parasitic organisms whose identification can potentially influence treatment. Ideally, the amplification of one, or a few, visually diagnostic RAPD fragments can be achieved which differentiate between sub-species or variants, without the need for extensive statistical analysis. In human diseases, for example, Adamson *et al.* (1993) employed the universal M13 primer as a random primer and produced specific profiles which permitted the identification of two sympatric sandfly species, responsible for the outbreak of two different forms of *Leishmaniasis*, allowing a fast, appropriate medical response. Similarly, Steindel and co-workers (1994) carried out a RAPD-PCR study on Trypanosome strains, which indicated the value of RAPD analysis to the study of parasites where largely unexplored genetic variation may have important bearing on the complexity and diversity of diseases.

Again in the field of parasitology, Cenis (1993) used RAPD-PCR to identify four major species of the *Meloidogyne* nematode, screening twenty-two primers in

the process of differentiating the species, allowing the demography of the species to be examined in greater detail. In a more down-beat report, Raich *et al.* (1993) tested four primers with the intention of differentiating species of the *Culicoides* midge, the vector responsible for bluenose fever in sheep and cattle, but detected similar RAPD profiles for each species. However, they correctly recognised the probability that further testing with a greater number of primers may well have produced more telling results.

In the same year, Perring *et al.* (1993) employed RAPD analysis as part of a programme to distinguish what was believed to be a variant of whitefly *Bemisia tabaci*, newly introduced into America and morphologically indistinguishable from "Type A" *B. tabaci*. Seven primers were screened and generated unique amplification products for each of the two types. When collated with other data on allozymes and mating behaviour, the recently introduced variant was indeed recognised as a new species of whitefly. As a final example of the utility of the RAPD technique as a method of generating species specific markers, Frey and Frey (1995) utilised RAPD profiles to establish a molecular identification key for six species of scale insect (*Quadraspidiotus* spp.), facilitating the assessment of the field specificity of pheromone traps.

RAPD analysis is not, of course, limited to invertebrate studies, and has also proved valuable in many other fields, including forestry. For example, accurate estimates of diversity between and within populations of trees are considered to be prerequisites for the optimisation of sampling and breeding strategies. Chalmers *et al.* (1992) identified population specific RAPD markers in *Gliricidia sepium* and *G.*

maculata and recommended RAPD analysis as a cost-effective method for precise and routine evaluation of variability within populations. Remaining in the plant kingdom, in soft fruit breeding, Graham *et al.* (1994) successfully used ten primers to differentiate ten raspberry cultivars. The relationships between the cultivars were examined using phylogenetic trees derived from similarity data, and the results compared with those produced from the published ancestry of the cultivars. The two phylogenies showed broad agreement. This accord between RAPD data and other classes of data, either genetic or morphological, is vital if the technique is to be accepted as part of the researchers armoury. A number of examples of parity between RAPD data and isozyme data exist e.g. Tibayrenc *et al.* (1993) researching the species phylogeny of protozoa; Heun *et al.* (1994) studying the relationship between successions of the wild oat *Aver sterilis*; Isabel *et al.* (1995) calculating gene diversity estimates in black spruce and Castagnone-Sereno *et al.* (1994) analysing relationships within the *Meloidogyne* nematode genus. Furthermore, Naish *et al.* (1995) reported that multilocus DNA fingerprinting and RAPD data revealed similar genetic relationships between strains of Cichlid fish. Whilst the multilocus fingerprinting produced greater genetic distances between the strains, the authors noted the technical ease and superior statistical robustness of the RAPD approach and data.

Since its conception, the RAPD technique has expanded its portfolio of applications as more researchers have utilised its capabilities. Ender *et al.* (1996) probed Southern transferred RAPD fragments of the waterflea (*Daphnia* spp.) and successfully identified SSRs (microsatellite) loci, which are rapidly gaining

importance as single-locus DNA markers in population genetics. This RAPD based method of identifying SSRs is applicable to anonymous genomes, avoids genomic library construction, screening, and the need for larger amounts of DNA. In addition, Bagley *et al.* (1997) employed RAPD amplification in conjunction with SSCP (Single Strand Conformation Polymorphism) analysis to detect anonymous potentially polymorphic loci in nuclear DNA which can then be used for further population genetic analysis.

The application of RAPD profiling has also been employed to determine last male sperm precedence, for example, in the damselfly *Calopteryx splendens xanthostoma* (Hooper and Siva-Jothy 1996). This involved comparing band-matching coefficients between putative parents and individual fertilised eggs. This practice has, however, come under criticism due to the appearance of non-parental bands appearing in the offspring of known primate pedigrees (Riedy *et al.* 1992).

RAPD markers have also been successfully applied to the sexing of birds. Lessells and Mateman (1998) screened RAPD primers against known sex birds from a number of species and identified primers which produced species specific bands with females, with a high degree of accuracy. This application of the RAPD technique could theoretically be applied to many organisms.

RAPD markers can be linked to specific chromosomal regions, and have been used to isolate genes of interest without requiring a genetic map of the entire genome. For example, RAPD markers located in regions linked to nematode resistance genes in tomatoes (Klein-Lankhorst *et al.* 1991), downy mildew resistance

in lettuce (Paran *et al.* 1991), and virulence characteristics in soybean cyst nematodes Li *et al.* (1995) have been identified and utilised in breeding programmes.

In the field of conservation ecology, genetic diversity data derived via RAPD-PCR from endangered species are allowing educated decisions to be made in respect to the breeding strategies and land-use management necessary to maintain a healthy level of genetic mixing in populations with a limited base-line of variability, in both plants and animals (e.g. Rossetto *et al.* 1995; Gibbs *et al.* 1994).

RAPD analysis has also proved a useful tool in the study of invertebrate population structure, which is more directly relevant to this thesis. An early study on the use of RAPD analysis was published in 1992 by Kambhampati *et al.* who reported work in discriminating mosquito populations. The Kambhampati *et al.* (1992) study was mirrored the same year by Ballinger-Crabtree *et al.* (1992), who also successfully used RAPD data to differentiate and identify mosquito populations based on patterns of genetic variation. The ability to identify and analyse potential disease carrying vectors can have a profound effect on epidemiological research.

Since then, a number of further studies have utilised RAPD-PCR to study population structure via the analysis of levels of variation. For example, Caswell-Chen *et al.* (1992) successfully used RAPD markers to distinguish between the sympatric nematode species *Heterodera schachtii* and *H.cruciferae*. Whilst inter-specific RAPD studies have been criticised due to questions regarding band homology (see Section 4.1.2 this chapter), this study also applied RAPD data to distinguish between intra-specific populations, analysing relationships via nearest neighbour hierarchical cluster analysis. Further RAPD work on nematode populations

has been carried out by, amongst others, Hahn *et al.* (1994) and Folkertsma *et al.* (1994), in Sri Lanka and the Netherlands respectively, and has proved informative in differentiating populations and providing information on historic invasion routes. All these researchers recognised the power that RAPD-PCR has to produce genetic information with these very small invertebrates, particularly where species specific sequence data may be limited or lacking altogether.

In two studies on very different organisms, but with comparable aims, Stiller and Denton (1995) employed RAPD markers to study the genetic history and current population structure of invasive cordgrass, in Willapa Bay, Washington, whilst Williams *et al.* (1994) elucidated on the possible geographic origin of an introduced insect pest *Listronotus bonariensis* by analysing data from two random primers. Both studies used statistical analysis of the variation in RAPD markers to indirectly assess likely historical population genetic structure.

In a geographically wide-ranging study, Puterka *et al.* (1993) studied the genetic diversity and phylogenetic relationships among global populations of the aphid *Diuraphis noxia*. Populations were analysed via 69 polymorphic marker bands amplified by seven primers and it was found that all populations could be distinguished with this method. In addition, there was near perfect agreement in the data derived by allozyme and RAPD-PCR analysis.

The lengthy, and ever increasing list of RAPD studies on invertebrate species includes, amongst others, the Mediterranean fruit fly (Haymer and McInnis 1994; Haymer *et al.* 1997), the amphipod, *Corophium volutator*, (Wilson *et al.* 1997), several aphid species (e.g. DeBarro *et al.* 1995; Vanlerberghe-Masutti and Chavigny

1998); and the grape phylloxera (Fong *et al.* 1995). Notably, there are no RAPD based spider population studies currently in the literature, although Hettle *et al.* (1997) published a paper outlining the technique's potential for spider based studies. Surprisingly though, these authors visualised the RAPD fragments via autoradiography, a step which complicates this essentially simple technique a great deal.

These cited papers provide a brief overview of the potential the RAPD technique has to detect polymorphisms in a wide array of organisms, and why it has become a part of most molecular ecologists tool box - fast, effective and with relatively low running costs. Exit Dr Jeckyll, enter Mr Hyde.

4.1.2. Criticism of the RAPD technique

Despite its undoubted popularity as a tool in molecular biology, the RAPD analysis approach is not without its critics (e.g. Black 1993; Kreitman and Wayne 1994; Lynch and Milligan 1994). There are two avenues of criticism - theoretical and practical.

One, if not *the*, major drawback of RAPD data relative to allozyme, or other co-dominant data (such as microsatellite data) in the analysis of population genetic structure, is that the majority of polymorphisms revealed by RAPD segregate largely as dominant markers. Williams *et al.* (1990) and Fritsch and Reisberg (1992), for example, found at least 95 % of RAPD fragments behaved as dominant markers with only the remaining 5 % behaving codominantly. The dominance of bands precludes zygosity inference and hence the direct estimation of allele frequencies. That is to say,

given a present allele (A) and absent allele (a), a fragment will be amplified in both the homozygous (AA) state and the heterozygous (Aa) state. Only the frequencies of null homozygotes i.e. the absence of a band on a gel (aa), can be accurately assessed. This limitation causes problems in applying traditional population genetic statistics to RAPD data (e.g. Wright's F_{ST} values) which rely on knowledge of allelic frequencies, and leads to a bias in estimates of population genetic parameters. However, methods to reduce this bias have been presented and will be discussed shortly.

Further criticism of RAPD analysis stems from the fact that the amplified sequences are of unknown genomic origin, and subsequently there is no way of ascertaining, without carrying out further analyses, if apparently homologous fragments are in fact the same DNA sequence (and not different sequences which simply appear the same size on a gel). Recent work, involving restriction digestion of RAPD fragments (which would identify sequence differences in apparently homologous bands), was carried out in an attempt to quantify the extent of the problem, and reported that 90 % of fragments generated by RAPD-PCR were homologous *between* species in a genus. The paper states that within a species (i.e. population level studies) the percentage should therefore not be lower (Reisberg 1996). However, despite those results, van de Zande and Bijlsma (1995) stress the limitation of RAPD-PCR in relation to phylogeny reconstruction and strongly suggest restricting studies to sibling species only. This recommendation was based on hybridisation experiments of RAPD bands in *Drosophila* spp. which demonstrated that fragments of similar length were not always derived from corresponding loci even in closely related species. This casts doubt on a number of studies, including

that of Comincini *et al.* (1996), who used RAPD data from eight primers to examine the systematic relationship between five Cervid (deer) taxa. As well as reaching broad agreement with results from previous studies which had employed different methods, they arrived at new possibilities within the examined taxa with their RAPD data. These new possibilities may well have been based on incorrect homology assumptions across taxa. Noticeably fewer researchers have applied RAPD-PCR to phylogeny reconstruction as these reservations have been expressed.

Finally, a third theoretical criticism levelled at the RAPD approach, again in relation to phylogenetic analysis relates to the genomic origin of the fragments. As fragments are thought to be amplified from both coding and non-coding regions of the genome, they may be subject to very different evolutionary pressures, whilst the unweighted pairgroup method of arithmetic averages (the commonly used UPGMA analysis to create dendrograms of phylogenetic relationships based on similarity) assumes a consistent universal molecular clock. This is indeed an important, if moot, point for phylogenetic analysis, (which as mentioned should not be attempted using RAPD data at levels deeper than genus), but for studies of contemporary genetic variation within a species, it has no real bearing, since our aim is only to observe current patterns of DNA variation and not calibrate or conjecture about deeper evolutionary relationships.

The second avenue of criticism raised in regard to the RAPD technique is technical. Perhaps the greatest criticism is the issue of reproducibility of results between laboratories. Several independent studies have been carried out which have indicated that inadvertently changing reaction parameters or components can greatly

affect the RAPD profiles generated (e.g. Yu and Pauls 1992; Schierwater and Ender 1993). However, most practitioners of RAPD-PCR agree that these criticisms can be met by careful attention to maintaining consistent protocols and laboratory practices (e.g. Grossberg *et al.* 1996) and that the benefits of a readily available supply of polymorphic genetic loci counterbalances the effort required in what simply amounts to good-housekeeping.

4.1.3. Analysis of RAPD data

The statistical analysis and interpretation of RAPD data is currently an area in great flux, and a number of different approaches are present in the literature. Naturally, the first step is to document the RAPD profiles following gel electrophoresis, by scoring for the presence or absence of bands. However, even this preliminary step has a number of possible permutations! For example, bands may be scored simply as present or absent, regardless of the strength of staining, or some account can be taken of their intensity. Most authors tend to screen primers to identify those which consistently produce strong profiles (i.e. brightly staining bands) e.g. Stewart and Excoffier (1996). As a complication, some researchers choose to analyse all fragments, whilst others score only those in a certain molecular size range. Stewart and Porter (1995) for example, scored only bands in the middle molecular weight range. In comparing duplicate reactions they observed that occasionally high molecular weight bands would be replaced by several low molecular weight bands, resulting, they concluded, from nested inverted repeat sequences (primers sites within a large fragment). This did not appear as a problem in the course of this study, and all

bright, reproducible bands were scored. Ultimately, whichever scoring route is taken, a matrix of binary presence/absence (scored 1 or 0) data is generated for each sample which can then be analysed in a number of ways. Before analysis however, a number of assumptions are made regarding the RAPD data and must be borne in mind;

(i) All RAPD loci show complete dominance (meaning that the absence of a band indicates a recessive homozygote);

(ii) All loci possess only two alleles;

(iii) All populations are considered to be in genetic HWE (Hardy-Weinberg Equilibrium) i.e. the percentage of bands absent at a given locus will yield the frequency of the recessive homozygote, from which the frequency of the two alleles can be calculated;

(iv) Co-migration of RAPD bands indicates sequence homology.

Clearly few, if any, of these assumptions will be an entirely accurate reflection of the true situation (for example, as mentioned previously, some 5 % of RAPD bands are thought to be codominant), and due to this, the population parameters calculated from RAPD analyses must be treated cautiously. However, levels of genetic variation can clearly be detected, and steps can be taken to reduce the statistical bias of the population genetic parameters calculated from RAPD data.

Currently, the most widely used analysis in population level RAPD studies is the calculation of similarity coefficients between samples, to quantify the level of similarity between individuals or populations, followed by the generation of a graphical representation of the similarity data, either by creating a dendrogram or plotting a principle co-ordinates (PCO) diagram.

The calculation of similarity coefficients allows the combination of results from all primers, or the treatment of primers separately. The two most commonly used methods of calculating similarity are the Nei and Li (1979) coefficient and the Jaccard (1901) coefficient (e.g. Caswell-Chen *et al.* 1992). Lamboy (1994) argues that the Nei and Li coefficient has an advantage over Jaccards' in RAPD studies using closely related organisms or populations (as should always be the case given the misgivings over band homology and inter-specific comparisons), because in this case most of the similarity between RAPD profiles will be caused by shared positive bands, and the Nei and Li coefficient displays less percent bias than Jaccard in these circumstances. In reality, most researchers (e.g. Ballinger-Crabtree *et al.* 1992; Williams *et al.* 1994) perform several similarity algorithms with their RAPD data and compare and contrast results (on the basis that parity of results indicates robustness of data). Indeed, McNicol (1995) directly compared results from RAPD analysis with both methods and highlighted that in most cases there is often very little difference in outcome.

Dendrograms can then be derived from a similarity matrix via UPGMA (e.g. Fong *et al.* 1995), neighbor-joining (e.g. Haymer *et al.* 1997) or other tree generating methods, which produce a visual representation of the relatedness of individuals or populations. Dendrogram construction is a hierarchical procedure which assesses the similarity between all samples and constructs a branched tree to represent relationships. The UPGMA method is the simplest method of tree construction. It employs a sequential clustering algorithm, in which relationships are inferred in order of decreasing similarity i.e. the most similar individuals are joined and treated as a

single unit, which is then joined to the next similar, and so forth through all the samples.

An alternative method of partitioning and visualising variation between samples is to carry out PCO (principle co-ordinate) analysis e.g. Gabrielsen *et al.* 1997; Graham *et al.* 1997. PCO plots are also based on the matrix of similarity, but avoid any emphasis on hierarchical clustering. The PCO analysis presents a two-dimensional scatter plot of the three-dimensional distance matrix of the sample array, representing as closely as possible the original genetic distances among all the samples. This is feasible as PCO analysis works in such a way to partition most inter-specimen variance into the first few axes. The PCO approach has the benefit of allowing the human brain's well developed ability to assess spatial relationships detect patterns in the data (which can then hopefully be related to an intuitive division in the samples e.g. geographic location, habitat type, altitude, etc.).

The methods described above have been concerned with examining quantitative levels of *variation* between groups of individuals and representing the data in an easily interpretable form. However, as the applications of the RAPD technique have expanded and evolved, so concomitantly has the analytical theory. If more traditional qualitative population genetic parameters (e.g. direct estimation of allele frequencies to provide genetic diversity and F_{ST} estimates) are sought, RAPD data present several problems as alluded to earlier. The F_{ST} estimate is a particularly important statistic to calculate as it is a guide to the overall level of genetic subdivision among sub-populations (Wright 1978).

Lynch and Milligan (1994) were the first to address the statistical problem of fragment dominance in terms of generating estimated allele frequencies from RAPD data with reduced bias. As a result of their analysis, two major recommendations were presented; Firstly, 2-10 times more individuals should be sampled per RAPD locus than per co-dominant allozyme (or microsatellite) locus to reduce the fraction of the loci that will yield biased parameter estimates in diploid material (i.e. 20-100 individuals). Secondly, by scoring only markers which are not too common; $x < 3/n$, (n = sample size), more unbiased estimates of population genetic parameters can be achieved.

Hamelin *et al.* (1995) applied the Lynch and Milligan criteria in their analysis of populations of the white pine blister rust fungus and found that their results produced the most accurate estimate of observed heterozygosities when both genotypic and phenotypic RAPD data were compared.

However, this method in turn has come under criticism from Szmidt *et al.* (1996), who concluded that even by including these steps to remove bias, intrapopulation diversity parameters derived from the indirect RAPD frequency were much lower than similar parameters obtained from complete genotype information. They also questioned the Lynch and Milligan (1994) assumption that populations will be in HWE. Their work indicated that the frequency of homozygotes was substantially below that expected under HWE conditions. This result implies that analysis of diploid material would require an even larger sample size than that suggested by Lynch and Milligan (1994). They further point out that the restriction of data sets to uncommon markers was satisfied for only a few highly polymorphic loci,

and caused substantial overestimates of gene diversity. Moreover, the loci which are rejected may differ from population to population (Lynch and Milligan 1994) creating difficulties in meeting the criteria.

Despite these criticisms however, the Lynch and Milligan (1994) approach has been adopted by many researchers as it was the first serious attempt to deal with the theoretical limitations of RAPD data. Other statistical treatments have also become available to add robustness to RAPD analysis. For example, Excoffier *et al.* (1992) introduced AMOVA (Analysis of Molecular Variance) to calculate a F_{ST} analogue from a paired squared distance matrix of mitochondrial haplotype data, and then subsequently adapted the methodology to accommodate RAPD binary data (in populations with assumed levels of self fertilisation) using American Cranberries as the exemplar (Stewart and Excoffier 1996). The AMOVA approach analyses variation, segregating the sums of squares of phenotypic distances into components representing variation among individuals within populations, among populations within-groups, and among geographically proximate groups (e.g. Wilson *et al.* 1997). Huff *et al.* (1993) and Peakall *et al.* (1995) demonstrated the AMOVA as an analytical technique with which to discern regional and population differences in two independent studies on buffalograss *Buchloe dactyloides*.

Further refinement of the approaches to analysing RAPD data include the work of Martinez-Torres *et al.* (1997), who presented a new method for the assessment of nucleotide diversity from RAPD data principally suited for asexual organisms with particularly low levels of polymorphisms, in this case, aphids.

Despite the well documented criticisms highlighted in this introduction, the RAPD technique remains one of the most suitable for assessing variation relatively quickly in natural populations of organisms on which there is little available sequence data and small amount of initial template DNA.

In this study, RAPD analysis is carried out on populations of *L.tenuis* and *E.ovata* to assess levels of genetic variation at a relatively local scale (< 100 km) and to discern if population structure can be detected.

4.2. MATERIALS AND METHODS

4.2.1. RAPD-PCR amplification conditions

The optimised RAPD reaction conditions as reported in Section 2.7.3 were employed.

4.2.2. Screening of primers

Screening a number of primers was carried out to identify those which highlighted polymorphisms between individuals, which could then be used with the sampled populations. Screening was carried out with the previously optimised conditions (Section 2.6.3), precluding the necessity of having to optimise reaction conditions for each primer individually, thereby increasing the speed of the method. To further accelerate the screening process a two level screen was employed.

In the first level, bulked DNA extracted from ten *L.tenuis* from Boghall (1 µl DNA from each genomic extraction plus 90 µl SDW) was used as the template against which 60 primers (Operon kits AF1-20, AR1-20 and B1-20) were screened. In the second level screen, primers which produced several distinct bands with the pooled DNA were then screened against a small subset (5 to 10) of individuals from Boghall, to allow an accurate assessment of the profiles and their polymorphic status. Two primers which gave no amplification with the pooled samples were also tested against individuals to ensure lack of amplification was not due to any inhibitory effects of bulking the DNA. A "good" primer was subjectively designated as one which produced consistent results and highlighted polymorphisms between

individuals. The same criteria were used with *E.ovata*, with 25 primers screened (Operon kit H1-20 plus selected others). The primer screening is summarised in Appendix 8.1.

4.2.3. Sample Populations

E.ovata

DNA samples from 48 individuals from three sites (Blackford Hill, Invergowrie and Spotte) and 28 individuals from Phantassie, all collected in July 1997, were amplified with five selected primers (See Figure 3.1 for geographic location of sampling sites).

L.tenuis

DNA samples from 48 individuals from three sites (Boghall, Phantassie and Spotte), all collected in June 1997, were amplified with five selected primers. (See Figure 3.1 for geographic location of sampling sites).

4.2.4. Scoring and statistical analysis of RAPD data

Gels were scored by eye for the presence or absence of fragments. This method is ultimately open to subjectivity in scoring, but consistent personal criteria were used. Each gel was scored twice. Although optical imaging programmes are commercially available to scan and score gels automatically, they are reportedly unable to cope with the slight gel inconsistencies and warping which may occur during electrophoresis (Grossberg *et al.* 1996) and the majority of researchers continue to score bands by eye.

Both quantitative similarity based measures, and more traditional qualitative population genetic measures, were calculated with the RAPD data for both the *L.tenuis* and *E.ovata* samples.

The level of genetic similarity was initially calculated by scoring for the presence or absence of bands using the pooled data from the five chosen primers for each species via both the Jaccard, and the Nei and Li coefficients. Parity of results using the different coefficients suggested that clustering in the data was likely to be robust. However, the Nei and Li coefficient was chosen as the method of choice for the reasons outlined previously. The Nei and Li (1979) formula for calculating similarity is as follows:

$$\text{Similarity} = (2 \times N_{ab}) / (N_a + N_b)$$

Where N_{ab} = number of shared fragments between individuals a and b

N_a = number of scored fragments of individual a

N_b = number of scored fragments of individual b

The distribution of similarity within each population was also investigated by fitting a beta-distribution curve onto the similarity values. A beta distribution curve was fitted to the data in preference to a normal distribution because the possible similarity values fall in a restricted interval of values (i.e. zero - 1). A normal distribution may assign probability density to values outside this range (particularly as the *E.ovata* values are all approaching the upper limit), and thus plotting a beta distribution is more appropriate in this case (Derman *et al.* 1973).

Dendrograms based on the UPGMA clustering method, and PCO analysis scatter-plots, were also generated from the similarity matrix data. Whilst many authors present a PCO plot without further analysis (e.g. Arens *et al.* 1998), in this study a number of further statistical tests were carried out on the PCO data to consolidate the analysis and conclusions. A one-way ANOVA (analysis of variance) was carried out to estimate variance components within and between populations (using the site as the factor) for the first two PCO axes (which contain the largest proportion of variation). A Scheffe's multiple comparison test of the mean population variance was also carried out with the populations of both species to test statistically where differences are located amongst the population means, and how many differences there are.

Finally, qualitative genetic analysis was carried out in accordance with Lynch and Milligan's (1994) paper to produce data on both heterozygosity levels (to estimate genetic diversity within each population), and to produce an estimate of F_{ST}, giving an indication of the overall level of population subdivision.

The analyses were performed using the SIMSTAT (Provalis Research, Canada) and GENSTAT 5 (1987) statistical packages.

4.3. RESULTS AND DISCUSSION

Despite its practical and theoretical drawbacks (as discussed, something of a compromise in terms of classical genetic theory) RAPD analysis clearly identified polymorphic genetic markers both within and between spider populations. Although the marker bands are anonymous, and their specific interpretation is open to debate, they are clearly loci generated from spider DNA and have proved informative for the purposes of this study. Indeed, RAPD analysis is an ideal tool for surveys of natural populations where there are only small amounts of biological material for analysis, little sequence information is known on the species in question, and a general evaluation of variability is being conducted at the population level. All these conditions apply to this initial study of spider populations. Nei (1973) writes, "...in order to know a general picture of gene differentiation among subpopulations, a large number of loci that is a random sample of the genome should be used, including both polymorphic and monomorphic loci." Certainly, RAPD analysis fulfils those criteria.

4.3.1. Screening of primers

Screening of primers with bulked DNA samples did not prove problematic, with the profiles produced representative of profiles obtained from individuals i.e. all bands recorded from a bulked sample were also present in individuals. The results of this first round of screening 60 primers identified 11 primers which were amplifying clear profiles with *L.tenuis* containing between four and 12 bands. These were then used in a second round of screening against a number of individuals from one site to

identify which of these primers highlighted polymorphisms between individuals. Notably, the primers were not screened against spiders from different sites in an attempt to find population specific markers, as this would surely bias the results (cf. Gabrielsen *et al.* 1997, who selected primers which detected variation between populations of an alpine plant). Primers were chosen simply on their ability to produce clear results highlighting polymorphisms between individuals.

Ultimately, five primers for each species were chosen for population screening from those which amplified clear profiles. The primers chosen for *E.ovata* analysis, presented in Table 4.1, generated 51 scorable bands, 27 of which were monomorphic across all populations. The *L.tenuis* primers, presented in Table 4.2, generated 50 bands for analysis. In contrast, only five of the *L.tenuis* bands were monomorphic across all the populations.

Table 4.1. RAPD-PCR primers (Operon Technologies, USA) used with *Enoplognatha ovata* populations

		No. of bands scored		
Primer designation	Sequence of bases (5'-3')	Total	Polymorphic	% Polymorphic
OP-H1	GGTCGGAGAA	12	8	66
OP-H2	AGACGTCCAC	8	4	50
OP-H5	AGTCGTCCCC	9	8	88
OP-H7	CTGCATCGTG	11	6	55
OP-AR19	CTGATCGCGG	10	7	70
Total		50	33	66

Table 4.2. RAPD-PCR primers (Operon Technologies, USA) used with *Lepthyphantes tenuis* populations

Primer designation	Sequence of bases (5'-3')	No. of bands scored		
		Total	Polymorphic	% Polymorphic
OP-AF15	CACGAACCTC	12	10	83
OP-AR3	GTGAGGAGCA	9	9	100
OP-AR19	CTGATCGCGG	12	10	83
OP-B03	CATCCCCCTG	7	6	86
OP-B20	GGACCCTTAC	11	11	100
Total		51	46	90

Figure 4.2 presents an example of a RAPD reactions which was scored in the study. The full RAPD data sets for both species are presented in Appendix 8.2.

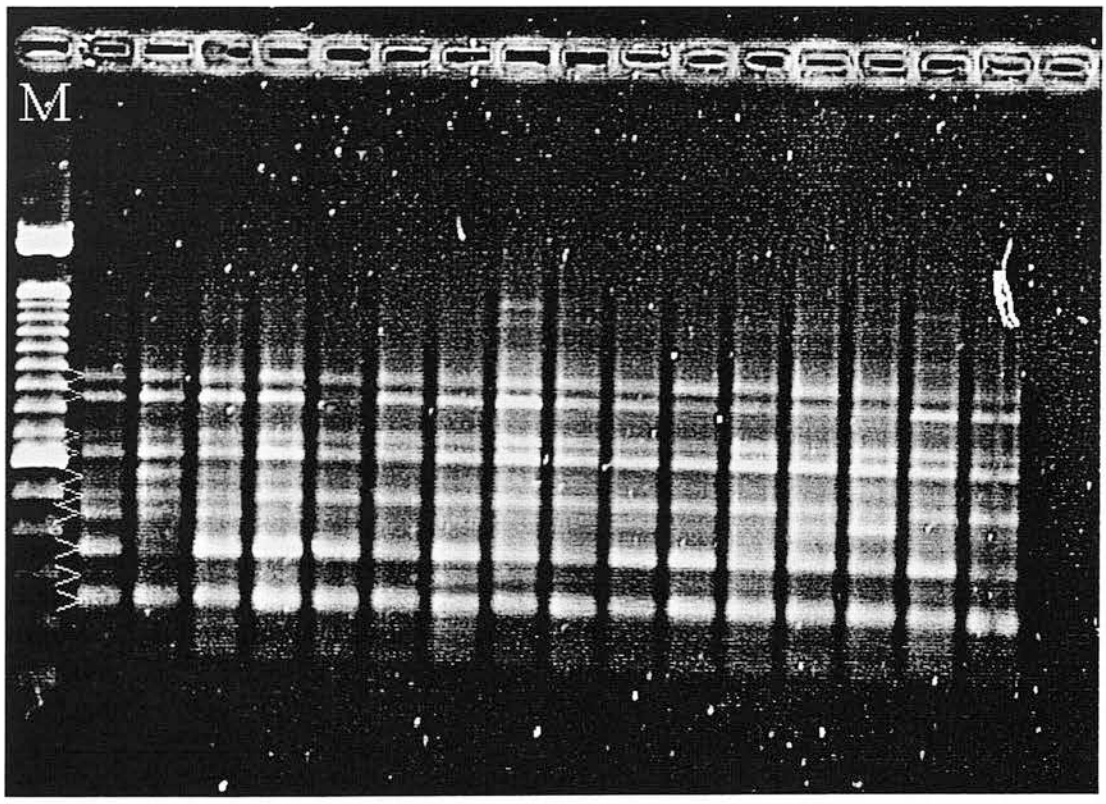


Figure 4.2. Example of a RAPD profile scored in the study. Sixteen *Enoplognatha ovata* from Phantassie with primer OP-AR19. The arrows highlight the 10 bands scored with this primer. M= molecular size marker.

4.3.2. Similarity matrices

The similarity data generated with the two distance measures produced highly comparable results, but the following analyses refer to similarity values generated via the Nei and Li coefficient only. In general, *E.ovata* individuals showed a very high degree of similarity with respect to the marker data analysed in this study. The most similar individuals (both from Spotte) were 98.6 % similar (such a high similarity may indicate consanguineous matings), while all individuals from the four locations were at least 83 % similar. *L.tenuis* individuals proved to be more genetically variable than those of *E.ovata* on the basis of the current data set. The most similar individuals in this species (again from Spotte) were 86 % similar, while all individuals from the three sites examined were at least 54 % similar. Notably, the similarity values are lower than those for *E.ovata*, despite the fact the *E.ovata* data set is for a larger number of individuals and includes an extra site (which one would expect to lower the degree of similarity).

As a method of highlighting the distribution of similarity levels within populations a beta distribution curve was fitted to each population, then a composite plot produced for all populations in both species (Figure 4.3). The distribution of variation is visually quite different between the two species, with a narrow base of variation in the *E.ovata* populations all tending towards the right hand side of the plot. The *L.tenuis* distribution has a more centralised, broader base of variation. A Mann-Whitney test was carried out to test statistically the differences of ranked distribution of similarity in the *L.tenuis* and *E.ovata* from Spotte, and indicated that the difference in distribution is indeed significant ($p < 0.05$). This emphasises that

differences in levels of genetic variation are present between *L.tenuis* and *E.ovata* populations from the same site (although admittedly the direct relevance is questionable given that different primers were used for each species).

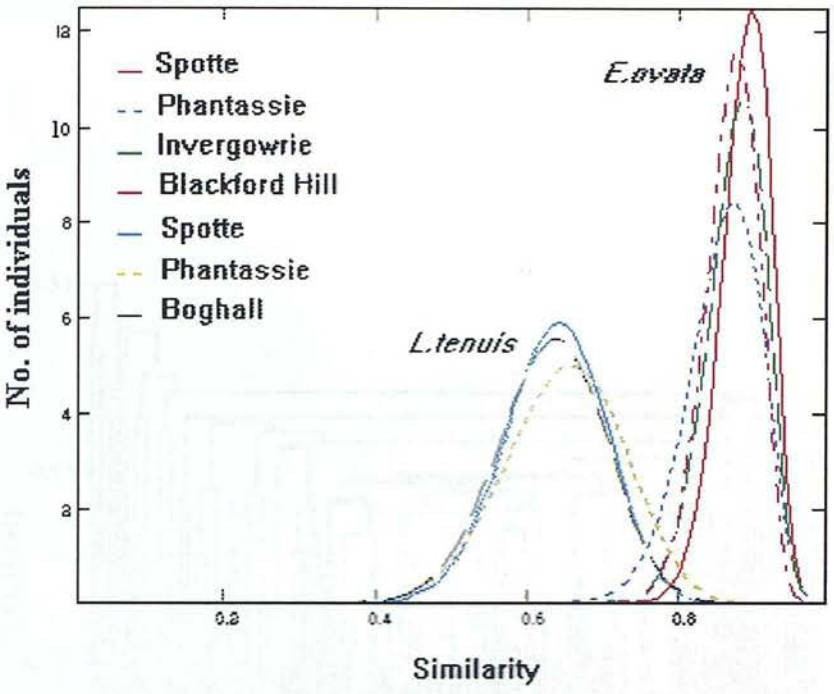


Figure 4.3. Beta distribution of within site similarities for populations of *Lepthyphantes tenuis* and *Enoplognatha ovata*.

4.3.3. UPGMA analysis

Due to both the high level of similarity between all individuals in each species (54-86 % *L.tenuis* and 83-99 % *E.ovata*), and the relatively large number of individuals from each population, the UPGMA dendrograms did not prove particularly useful as a population discrimination tool (Figure 4.4 and 4.5). What can be drawn from a global comparison of the two dendrograms, however, is the presence of a large central grouping in the *L.tenuis* dendrogram at around the 60 %

similarity level, indicating that a large proportion of the spiders belong to a single genetic group. In contrast, the *E.ovata* dendrogram has a number of more distinct clusterings, indicating a number of separate genetic groups. It is not immediately obvious however if individual spiders are grouped into their respective source populations.

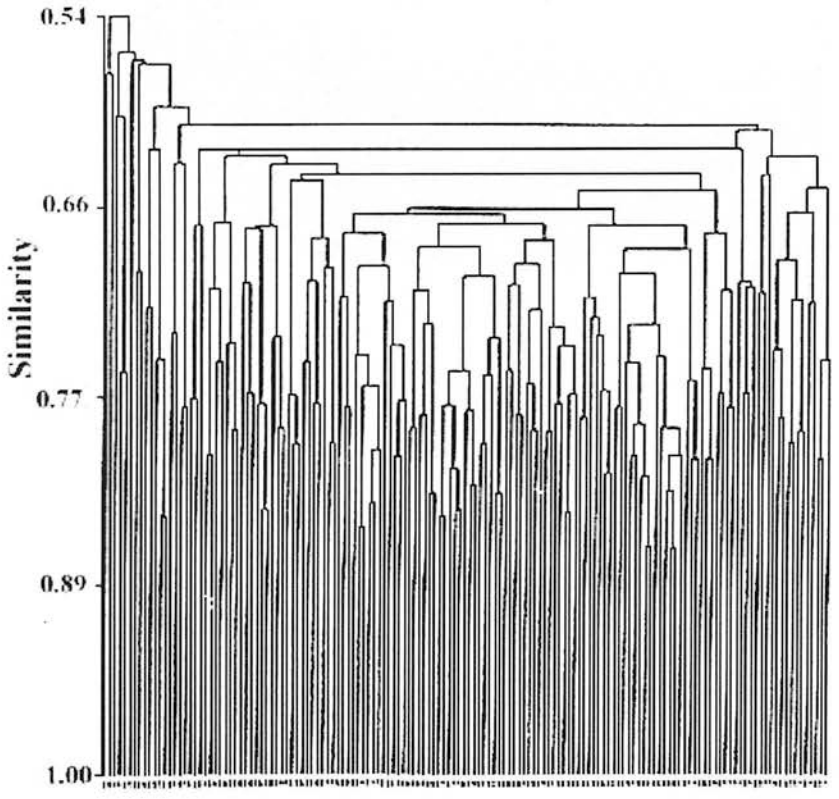


Figure 4.4. UPGMA dendrogram of *Lepthyphantes tenuis* from Spotte, Boghall and Phantassie

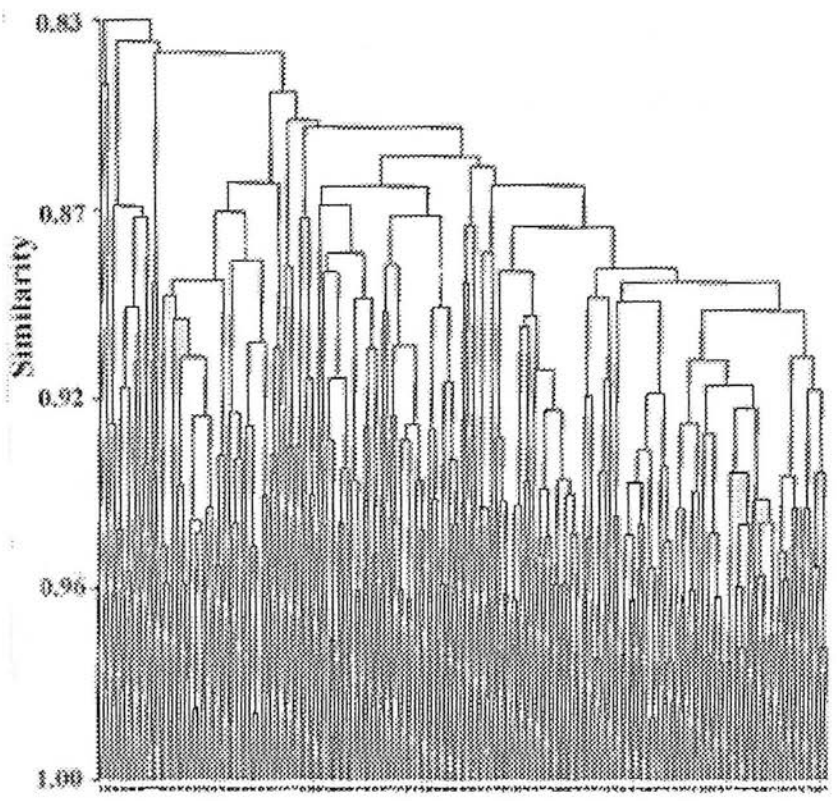


Figure 4.5. UPGMA dendrogram of *Enoplognatha ovata* from Invergowrie, Spotte, Phantassie and Blackford Hill

4.3.4. PCO analysis

In contrast to the UPGMA analysis, and despite the high level of intra-specific similarity, a structure can be detected in the first two *E.ovata* PCO axes which can be related to the geographical separation of the populations. The samples from Invergowrie, Dundee and Blackford Hill, Edinburgh (geographically the sites furthest apart) were relatively distinct along the first PCO axis, although no population was completely separated (Figure 4.6). In contrast to *E.ovata*, no such obvious geographical structure was evident in a visual inspection of the *L.tenuis* PCO plot,

although the Spotte samples did appear slightly clustered towards the top of the plot (Figure 4.7). It was surprising that the Spotte population should appear distinct from the other two, as it might be expected that Boghall, South West of Edinburgh, would show differentiation from Spotte and Phantassie, as the latter two are situated closer geographically.

The first two PCO axes are those which explain most of the variation in two dimensions. It can be seen that a greater proportion of the variation (17.4 %) is explained over the first two *E.ovata* axes than with *L.tenuis* (11.8 %), highlighting the greater separation in the *E.ovata* data (the greater the proportion explained in the first axes, the more pronounced the separations). However, it must be emphasised that a PCO plot very much produces an estimate of genetic distances between samples, and should only be used as an aid to understanding relationships, and not as a definitive guide. On the other hand, PCO analysis is not directly affected by the assumptions made about RAPD markers regarding zygosity, allele number and HWE status (Section 4.1.3. (i)-(iii)), which is in contrast to the calculation of heterozygosity levels and estimates of population differentiation. In this resepect PCO analysis is perhaps less prone to bias.

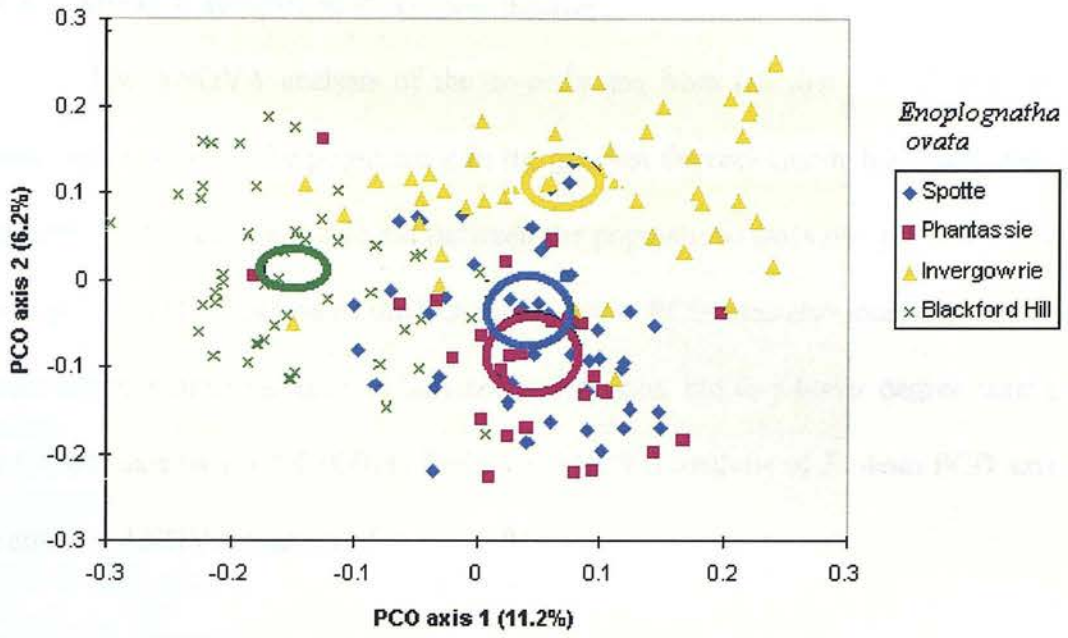


Figure 4.6. Principle co-ordinate analysis of *Enoplognatha ovata* similarity data.
The 95% confidence interval around the population means are shown in their respective colour.

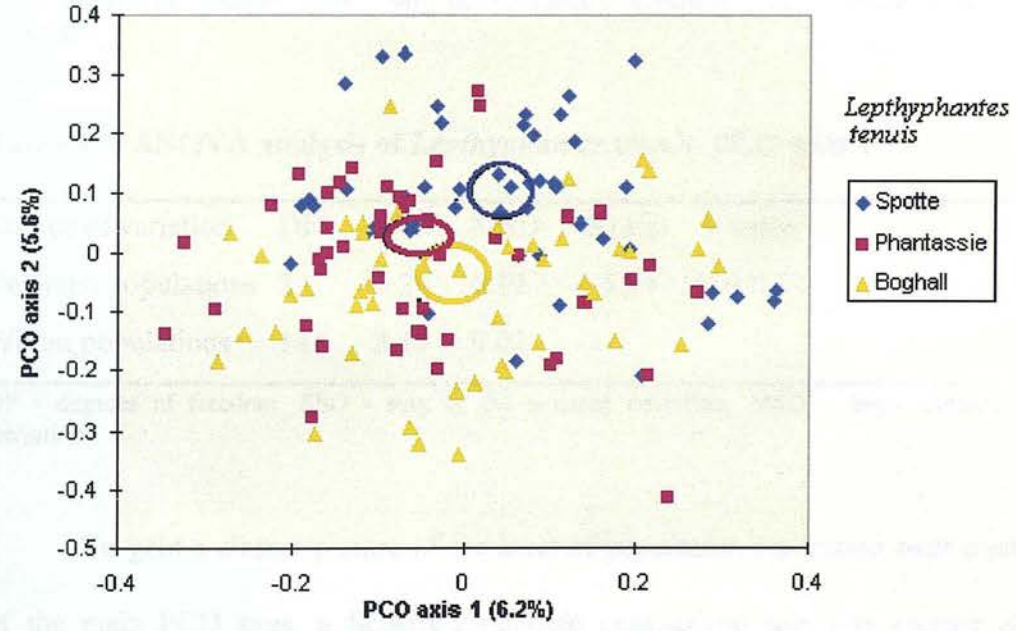


Figure 4.7. Principle co-ordinate analysis of *Lepthyphantes tenuis* similarity data. The 95% confidence interval around the population means are shown in their respective colour.

4.3.5. ANOVA analysis of PCO co-ordinates

The ANOVA analysis of the co-ordinates from the first two *E.ovata* PCO axis (which separate the populations to the greatest degree) clearly highlights that the majority of the variation is found between the populations (axis one $p < 0.0001$; axis two $p < 0.0001$). Analysis of the first two *L.tenuis* PCO axes also indicates that these axes attribute most variation to between populations, but to a lesser degree (axis one $p < 0.01$; axis two $p < 0.0001$) (Table 4.3 ANOVA analysis of *E.ovata* PCO axis 1; Table 4.4 ANOVA analysis of *L.tenuis* PCO axis 1).

Table 4.3. ANOVA analysis of *Enoplognatha ovata* PCO Axis 1

Source of variation	DF	SSD	MSD	F ratio	P value
Between populations	3	1.51	0.50	71.03	$p < 0.0001$
Within populations	168	1.19	0.01		

DF - degrees of freedom, SSD - sum of the squared deviation, MSD - mean squared deviation

Table 4.4. ANOVA analysis of *Lepthyphantes tenuis* PCO Axis 1

Source of variation	DF	SSD	MSD	F ratio	P value
Between populations	2	0.24	0.12	5.34	$p < 0.01$
Within populations	141	3.13	0.02		

DF - degrees of freedom, SSD - sum of the squared deviation, MSD - mean squared deviation

To gain a clearer picture of the level of population separation over a number of the main PCO axes, a Scheffe's multiple comparison test was carried out to compare population means within a species using data from the first five axes (which

encompass the majority of variation). Scheffe's comparison examines the significance of between site differences based on the mean PCO value for a particular axis.

In the case of the *E.ovata* populations, the separation of Blackford Hill from Invergowrie was supported in three of the first five axes ($p < 0.0001$, $p < 0.0001$ and $p < 0.05$) indicating strong differentiation. The Phantassie/Blackford Hill separation was also supported in three of the five axes ($p < 0.0001$, $p < 0.001$ and $p < 0.01$), again indicating strong differentiation. The Spotte/Blackford Hill and Spotte/Invergowrie comparisons were significantly different in two of the five axes ($p < 0.0001$ and $p < 0.05$). The other combinations of Spotte/Phantassie ($p < 0.01$), and Phantassie/Invergowrie ($p < 0.0001$), were significantly different in only one of the first five axes. Notably, however, all populations could be identified as statistically significantly distinct in a least one of the first five axes (including Spotte and Phantassie, the closest populations geographically).

Scheffe's multiple comparison of the *L.tenuis* populations reflects that Spotte is statistically significantly distinct from both Phantassie ($p < 0.01$ and $p < 0.0001$) and Boghall ($p < 0.0001$ and $p < 0.01$) in two of the first five axes. However, Phantassie and Boghall are not distinct across any of the five axes. From the analysis of the PCO plot, ANOVA and Scheffe's test data it would appear that Blackford Hill and Invergowrie are relatively genetically distinct *E.ovata* populations, whilst Spotte appears distinct from the other two *L.tenuis* populations. However, further analysis carried out reinforces the former supposition, whilst weakening the latter.

4.3.6. Heterozygosity values

Heterozygosity values (H) were calculated from the RAPD binary data as suggested by Lynch and Milligan (1994) to produce an estimate of gene diversity for each population (Table 4.5).

Table 4.5. Heterozygosity values (H) for *Enoplognatha ovata* and *Lepthyphantes tenuis* populations (See also Appendix 8.3 for bootstrap analysis)

E.ovata	(H)	*L.tenuis*	(H)
Spotte	0.2636	Spotte	0.3133
Phantassie	0.2604	Phantassie	0.3199
Invergowrie	0.2030	Boghall	0.3156
Blackford Hill	0.2719		

The heterozygosity values represent within population measures of genetic diversity, and clearly show that *E.ovata* populations have lower levels of within population diversity than *L.tenuis*. This would be expected given the hypothesised differences in gene flow and reinforces the results of the ANOVA analysis of the PCO co-ordinates. Interestingly, the Blackford Hill population has the highest level of heterozygosity of the *E.ovata* populations. It may have been expected that this most geographically isolated population would show the reverse, due to increased allele fixation in an inbreeding population (Chakraborty and Nei 1978). Perhaps the population is relatively recent or significantly larger than the others, and has not undergone many generations of inbreeding. The *L.tenuis* heterozygosity values were very similar across the three populations and were notably higher than any of the *E.ovata* population values.

The data for both species were also bootstrapped 300 times (creation of 300 pseudo-replicate data sets), and H values re-calculated, as a statistical method of gauging the "robustness" of the original values. Re-assuringly, the mean bootstrap estimates for all populations were within 0.01 of the originally calculated values (See Appendix 8.3 for a summary of the bootstrap analysis).

4.3.7. F_{ST} estimates

The average population F_{ST} value for the *L.tenuis* populations was calculated as minus 0.006, and across the *E.ovata* populations 0.053. Theoretically, F_{ST} has a range of values from zero (no genetic differentiation) to 1 (indicating the fixation of different alleles in different populations). However, the observed maximum in real populations tends to be substantially less than the hypothetical maximum. Wright (1978) suggested the following qualitative guidelines for interpreting F_{ST} values: 0-0.05 indicates little genetic differentiation; 0.05-0.15 indicates moderate genetic differentiation and values of > 0.15 indicate great differentiation. However, rather cryptically Wright (1978) also adds, "...differentiation is by no means negligible if F_{ST} is as small as 0.05 or even less". The negative value attributed to the *L.tenuis* populations highlights a complete lack of population substructure.

From the spider data it can be suggested (whilst accepting that the F_{ST} values were generated from dominant RAPD data and therefore contain a level of bias) that the F_{ST} values support the greater differentiation of populations in *E.ovata*.

The results from the above statistical analyses indicate what may have intuitively been expected, namely that populations of the frequent ballooner possessed less genetic structure than those of the more sedentary spider. A number of

explanations can be hypothesised to explain the differences in population genetic structure.

As reflected in the similarity data, the analysis of the spider RAPD data indicates that *L.tenuis* within Lothian show less similarity to each other on an individual level, yet were more similar overall, than *E.ovata*, as indicated by their lack of defined population structure. This apparent paradox can perhaps be explained in terms of increased allelic diversity. If a population contains immigrants from diverse genetic backgrounds, then the overall level of variation within that population will be high (i.e. a low level of homogeneity). If that scenario is then expanded to encompass the three *L.tenuis* populations (whilst assuming they also receive genetically diverse immigrants) this would lead to an unstructured genetic composition between populations i.e. all populations are then equally genetically diverse.

Alternatively, the genetic diversity could be a reflection that highly dispersive species are thought to be less likely to lose novel alleles, and should therefore possess more polymorphic loci and greater overall genetic diversity than sedentary species (Hamrick and Godt 1996). This is borne out by the population heterozygosity estimates (Table 4.5).

In contrast, the sampled *E.ovata* populations may have all arisen from a genetically impoverished stock in the relatively recent past (e.g. a population bottleneck) and hence show a large degree of similarity across populations at the individual level (83 %). However, genetic drift, caused by reproductive isolation, in the Invergowrie and Blackford Hill sites may have caused differentiation, as detected

in the PCO analysis, sufficient to allow the separation of these populations. This is supported not only by the heterozygosity values from within populations, (the *E.ovata* populations recording lower estimates than the *L.tenuis* populations), but also by the F_{ST} values and the Scheffe's test.

The above hypotheses are only a few of a number of possible explanations for the levels of variation recorded in the study, which will in truth, only be elucidated fully in time by additional studies. A further, if slightly more mundane, explanation of differences in levels of genetic variation between the species could arise from the fact that the primers chosen to screen *E.ovata* populations generated more monomorphic bands than the *L.tenuis* primers. This bias may have increased the level of similarity across all individuals. However, the degree of separation in the PCO plots, which should have been artificially reduced by the greater number of monomorphic bands, still reassuringly reflects what we know of the reproductive ecology of these spiders - greater population differentiation for the more sedentary species. Indeed, the higher proportion of monomorphic bands may be a reflection of a greater number of fixed alleles. Furthermore, the Lynch and Milligan (1994) calculation of heterozygosity only considers loci which occur at a frequency of less than 1-3/n (n = sample size), so in this instance, the potential bias of monomorphic bands is removed.

It must be noted that neutrality is being assumed in regard to the loci used in this study and whilst natural selection cannot be entirely discounted as a source of genetic variation, it is not considered a major factor. What is interesting to reflect on is a point raised, albeit in reference to a perennial plant, by Martin and co-workers (1997) - loss of genetic variation is thought to reduce the ability of populations to

adapt to changing environments and hence to survive. The *L.tenuis* populations analysed have high levels of genetic heterogeneity, which may reflect their life history strategy akin to that of a "weedy species" which can colonise and survive in a number of different habitats (Topping and Sunderland 1998). *E.ovata* in contrast, is more restricted in its habitat, and has lower levels of variation between populations.

As stated, the dispersive *L.tenuis* has a less clearly discernible population genetic structure visible in the PCO analysis across the three populations examined, although Spotte is distinct from the other two sites as revealed by the Scheffe's analysis. The heterozygosity and the F_{ST} values, however, indicate that there is little structuring of the *L.tenuis* populations. This lower level of genetic structure is not unexpected given the greater degree of gene flow which may be occurring in this ballooning species which would result in a constant homogenisation of the gene pool. Indeed, the spatial scale across which a species is genetically structured is thought to depend on two factors. The first is habitat discontinuity, the second, dispersal ability. *L.tenuis* has little or no habitat discontinuity in the British Isles given the range of habitats it colonises and the ubiquity it displays, and its potentially huge dispersal range due to ballooning would immediately suggest that gene flow is likely to have occurred in recent times across the geographic scale studied.

In contrast, the more sedentary *E.ovata* shows a more distinct population differentiation over a similar geographic scale, with the Invergowrie and Blackford Hill sites (approximately 100 km apart) showing almost complete separation from the other two sampled populations. Wright (1943) suggests that linear populations are more likely to undergo differentiation than multi-dimensional populations. This would

seem to be the case with the *E.ovata* populations studied, as all but the Blackford Hill sites were sampled along roadsides where suitable vegetation structure is available for these spiders. It could be argued that these sites are more geographically isolated, with Blackford Hill situated centrally in Edinburgh, and Invergowrie on the North side of the Tay estuary, and so a fair comparison is not being made with the *L.tenuis* populations. This is accepted. Ideally, sampling of *L.tenuis* would have been carried out at these locations, but was not practical. For this preliminary study of variation, geographic distances were measured from "as the crow flies" point-to-point distances.

Although far from clean-cut, these results could justifiably be taken as an indication of the geographic range at which the RAPD markers in this study can begin to identify the spatial scale of population size with *E.ovata*. Hastings and Harrison (1994) stress that whilst genetic data cannot be easily applied to determine rates of either population turnover or gene flow, especially since the two may produce similar patterns, gene frequency data can be very helpful in identifying the appropriate spatial scale for assessing populations. In the case of *L.tenuis*, further analysis will have to be undertaken to highlight the geographic scale at which populations can be differentiated. Undeniably, the survey for both species would benefit from the greater statistical strength of increased samples sizes and the number of bands scored, but this was not possible in this initial study.

However, the conclusions drawn from the RAPD analysis of *L.tenuis* and *E.ovata* in this study compare favourably with the survey of Loveless and Hamrick (1984), who collated allozyme data on the population genetic structure of plants in

relation to various aspects of life history. Plants with long range seed dispersal, and a widespread geographic range, akin to *L.tenuis*, were found to have high levels of heterozygosity within populations, but low levels of genetic structure among populations, reflecting the RAPD results presented in this study. Plants with limited seed dispersal, more akin to *E.ovata*, had a higher level of genetic structure and lower level of heterozygosity within populations, again reflecting the *E.ovata* results. Similar broad conclusions have been drawn for a number of fungi with differing dispersal abilities and life history strategies (e.g. Milgroom and Lipari 1995). Care must always be taken when directly comparing results from such different taxa, but in this case it is simply trends in genetic variation rather than specific values that are being compared.

Understanding what constitutes a population is important, as the degree of isolation among populations may be vital in deciding upon the appropriate scale at which to pursue land-management or conservation efforts. The ability to distinguish among alternative population structures - specifically distinguishing a collection of demographically isolated populations from a metapopulation in which migration or recolonisation may act to connect discrete populations, will play an important role. Hillis and Moritz (1996) emphasised that an understanding of population genetic structure must underlie sound species-management decisions. Given the degree of differentiation between the two populations at Blackford Hill and Invergowrie, it is probably fair to say that these *E.ovata* populations are demographically isolated and are receiving relatively few immigrants, leading to genetic drift and differentiation between populations. Indeed, the *E.ovata* populations appear to tend towards the

isolation by distance model of population structure, with genetic drift rather than gene flow the main determinant of population structure. However, it is also fair to say that some level of gene flow must be occurring, reflected in the relatively low F_{ST} estimate. Despite emphasising that *E.ovata* is quite sedentary, the species has managed to populate several states in America, despite only colonising in the 1950s. In comparison to the highly dispersive *L.tenuis* however, *E.ovata* keeps its eight feet firmly on the ground.

Reflecting on the *L.tenuis* data, it can be hypothesised that the sampled populations are perhaps more akin to members (demes) within a larger metapopulation, with large amounts of colonisation and dispersal occurring. With that in mind, it would be interesting now to increase this study to include a larger number of populations from both within Britain and abroad, to examine at what geographic scale *L.tenuis* populations can be clearly differentiated, and whether the *E.ovata* findings can be repeated and refined with further samples. As stated in Chapter Two, a larger study had been planned originally, with some 600 spiders from each species collected over a broader geographical range, but was curtailed by technical problems which rendered the samples unfit for RAPD analysis (freezer failure). Without further studies it would be unwise to try to predict what form the *L.tenuis* metapopulation may take. It would seem feasible to postulate that *L.tenuis* populations (again, at least at the geographic scale studied) are existing as a patchy metapopulation - where the locations are so strongly inter-connected by dispersal that local extinctions seldom occur.

Alternatively, by sampling areas of set-aside it may be possible to examine whether these areas are acting as longer lasting genetic pools in relation to surrounding fields, as hypothesised in the mainland-island metapopulation scenario (Harrison and Hastings 1996).

In terms of modelling the position of set-aside in the agricultural landscape, the results from these initial experiments tend to validate the existence of a (unstructured) metapopulation structure in *L.tenuis* as proposed by Topping and Sunderland (1994) and would therefore by default support the addition of non-rotational set-aside into the agroecosystem which they have predicted will raise the number of these beneficial predators in the surrounding arable fields.

4.4. Summary

Whilst the limitations and practical problems of RAPD-PCR must be borne in mind, following optimisation, the RAPD technique was a fast and reliable method of generating DNA fingerprints from individual spiders. The scoring and analysis of 50 and 51 RAPD bands generated from a set of five primers (10-mers) for *E.ovata* and *L.tenuis* respectively, produced data which when analysed via PCO reflected what is known of the life history and dispersal ability of the two species. *L.tenuis* populations within East Lothian appear to exist in genetically unstructured populations, whilst the less dispersive *E.ovata* tends towards an isolation by distance model of population structure with genetic drift rather than gene flow probably the main defining factor in population structure. These studies can be used as a framework on which to base further studies, which are required to elucidate fully spider spatial dynamics and population structure.

5. ANALYSIS OF SPIDER RIBOSOMAL DNA

5.1. Introduction

Structurally, in arthropods and all other eukaryotes ribosomal DNA (rDNA) is organised in clusters of tandem arrays, typically several hundred repeats in length (see Long and Dawid 1980 for a review). Each individual repeat unit contains both coding genes and spacer regions.

There are two internal transcribed spacer (ITS) regions present within each repeat, namely the ITS1 and ITS2, which separate the 18S, 5.8S and 28S rRNA genes. In addition, the intergenic spacer (IGS) separates each individual repeat unit (Figure 5.1). The IGS is itself comprised on the non-transcribed spacer (NTS) and the external transcribed spacer (ETS). The spacer regions are either not transcribed (the NTS) or are spliced out of the final mature rRNA. Typically, the arrays of rDNA repeating units occur at several sites both within and among chromosomes, at the nucleolar organising regions (Beckingham 1982).

The ITS region is characteristically not internally repetitive in terms of sequence (although McLain *et al.* 1995 did find some evidence of repetition) and comparisons between different *Drosophila* species have shown that at least half of the region evolves at a rate similar to the neutral rate of nucleotide substitution (Schlotterer *et al.* 1994).

Each repeating array is transcribed by RNA polymerase I, giving rise to a single primary transcript, the 45S pre-rRNA molecule. This molecule is processed further by the cleavage and removal of the spacer regions, liberating functional RNA, which in turn combines with ribosomal proteins to form ribosomes, the organelles

responsible for directing protein synthesis from messenger RNA (Beckingham 1982). The "S" descriptor of the ribosomal genes refers to the Svedburg coefficient, a measurement of sedimentation rate. Notably, since protein synthesis is obligatory for life, ribosomes, and hence rDNA arrays, are universally present in cells.

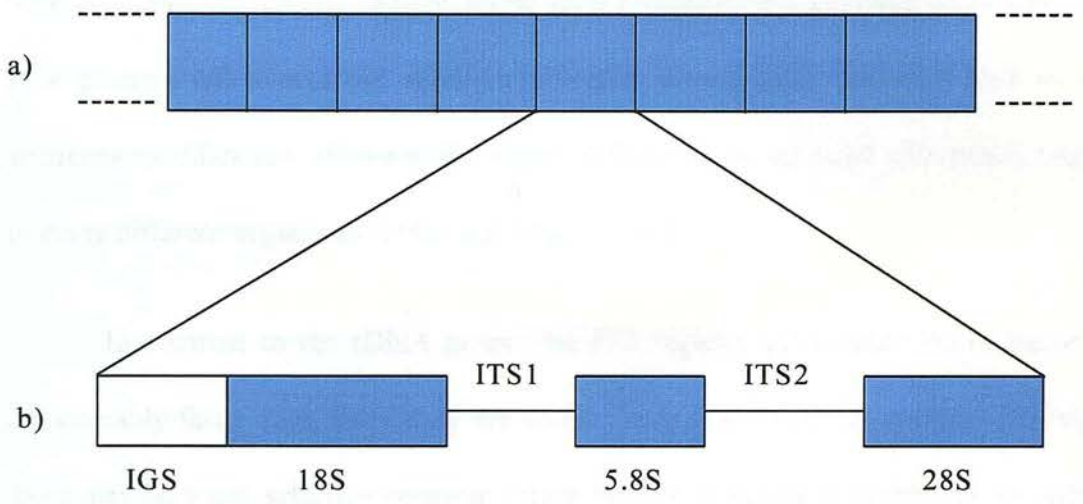


Figure 5.1. Diagram of the organisation of eukaryotic rDNA.
a) rDNA is comprised of repeated units
b) A single repeat unit consists of genes for the 18S, 5.8S and 28S rRNA. The genes are separated by spacer regions; the IGS, ITS1 and ITS2. The regions are not drawn to scale.

5.1.1. rDNA as a molecular marker

Due to its functional necessity, and hence ubiquity, rDNA has been readily adopted by researchers as a DNA marker system and has proved useful in gaining insights into a number of biological issues - ranging from the phylogenetic relationships between disparate taxa, to higher resolution population level studies (see Hillis and Dixon 1991 for a comprehensive range of applications). This utility across such a broad range of biological hierarchies is possible due to the variation in

rates of evolution of the different regions within the repeat units. The genes themselves are highly conserved due to their functional constraints, and undergo base changes at a relatively slow rate, making them ideal markers for broad phylogenetic analyses. Concomitantly, because of the conservation of sequence, the genes present themselves as ideal targets for amplification with so called "universal" PCR primers. That is to say, the coding regions retain such a conserved nucleotide sequence that PCR primers will anneal and allow amplification across most taxa with little or no sequence modification, allowing this region of DNA to be accessed with relative ease in many different organisms (Hillis and Dixon 1991).

In contrast to the rDNA genes, the ITS regions accumulate mutations at a considerably faster rate, since they are under fewer functional constraints (although there may be weak selective constraint since blocks of sequence homology do occur between closely related species (e.g. Wesson *et al.* 1992; Schlotterer *et al.* 1994; Section 5.3.4.7 this chapter). This rapid inter-specific sequence divergence between transcribed spacers was first demonstrated by Brown and co-workers (1972) for the frog sister species *Xenopus laevis* and *X.mulleri*. Whilst their ribosomal coding regions were virtually identical, the transcribed spacers were highly divergent.

As a result of reduced selection pressure and the subsequent level of variation present, ITS regions have been applied to the study of phylogenetic relationships among closely related taxa, and have even been applied to population level studies (e.g. Wesson *et al.* 1993; Vogler and DeSalle 1994). Amplification of these more variable regions is self-evidently facilitated by the fact they are flanked by the "primer

friendly" highly conserved coding genes in the array. A further important benefit of working with rDNA is that PCR-induced errors are held to become less important as the number of target template molecules increases, and therefore, moderately repeated DNA sequences - such as rDNA arrays - are less prone to *Taq* polymerase nucleotide mis-incorporation errors than single copy genes (Beven *et al.* 1992).

5.1.2. Concerted evolution of rDNA repeats

As ribosomal DNA regions in a number of organisms were characterised, a particular phenomenon became apparent. The multiple copies of the ITS regions present in each tandem array were not evolving independently, but seemingly in concert i.e. there was surprisingly low variation among the ITS regions in rDNA arrays both within individuals and throughout a species (whilst closely related species often have distinctive ITS sequences). This was indicative of the homogenisation of these multiple copies within an individual, and within a species.

The phenomenon of homogenisation has been termed "concerted evolution" (Zimmer *et al.* 1980). It is believed that homogenisation is brought about either by stochastic (random) cellular events such as the unequal crossing over of genetic material, either along (Schlotterer and Tautz 1994) or between chromosomes (Dover 1982; Worton *et al.* 1988), and/or by non-stochastic (biased) processes such as gene conversion. Unequal crossing over may occur either between two sister chromatids of a chromosome during mitosis of a germ-line cell, or between two homologous chromosomes at meiosis. It is a reciprocal recombination process creating a sequence

duplication in one chromatid or chromosome and a corresponding deletion in another (Nagylaki 1984).

Notably, unequal crossing over can produce fluctuations in the number of repeats in a gene family. In *Drosophila melanogaster* for example, a large deletion of rRNA genes was found to result in a "bobbed" mutant which in homozygous form manifested slow growth, low fertility and low viability. This could be reversed by an increase in the number of rRNA genes (Ritossa and Scala 1969; Schalet 1969). Gene conversion is, in contrast, a non-reciprocal recombination process, in which sequences interact in such a way that one is converted (replaced) by the other. As an explanatory mechanism for concerted evolution, it appears to have several advantages over unequal crossing over. Firstly, gene conversion does not cause a change in the number of gene repeats (which as mentioned can prove costly in terms of survival). Secondly, gene conversion can act as a correction mechanism, not only to tandem repeats, but also on dispersed repeats (Nagylaki and Petes 1982). Finally, gene conversion can have a preferred direction (hence "biased"), and a small disparity in the sequence composition of a repeat can have a large effect on the probability of fixation.

In addition to the homogenisation of repeats within individuals, to reach fixation (i.e. presence in all individuals) concerted evolution requires the sexual spread of mutations to all individuals in the population. Therefore, the effects of random genetic drift and population size have also to be considered as integral

components driving concerted evolution, as these factors will heavily influence the homogenisation of a repeat.

It must be noted that whilst unequal crossing over and gene conversion are considered the most probable cellular mechanisms which lead to homogenisation of multi-gene families, other processes such as DNA slippage and transposition are also capable of homogenising repeats. These are considered less likely candidates however, and the reader is referred to Li (1996) for descriptions of these cellular events.

The DNA turnover mechanisms which cause the phenomenon of concerted evolution have been collectively termed "molecular drive" (Dover 1982). They can bring about a long term change in the genetic composition of a population with respect to a given family of sequences, not unlike the effects of natural selection and genetic drift. However, the exact extent to which the various mechanisms are involved, and what the effect on evolutionary fitness the homogenisation of these repeated regions has, are not clear. It can be assumed that homogenisation is not deleterious since the phenomenon has been recorded in many species, and indeed conversely, it may be a mechanism for maintaining and proliferating a "fit" spacer region which allows for the specific patterns of folding required for effective processing of the 45S pre-rRNA. The number of rDNA repeats may also be of some importance, given the detrimental effect reduced array number had on the "bobbed" *Drosophila melanogaster* mutants.

The phenomenon of concerted evolution has been reported in the rDNA array of several arthropod species. For example, low ITS sequence variation has been found in populations of Anopheline mosquitoes across the species range (Fritz *et al.* 1994); species wide homogeneity of ITS2 sequences has been found in the spider mite *Tetranychus urticae* (although intra-individual studies were not undertaken (Navajas *et al.* unpublished)); and very low levels of variation were recorded in four populations the Cassava Green Mite from around the globe (Navajas *et al.* 1994). Similarly, Fenton *et al.* (1997) analysed the ITS1 region of *Cecidophyopsis* mites and detected little intra-specific variation, whilst low levels of variation have been reported in the genus *Drosophila* (Schlotterer *et al.* 1994) and in strains of the hymenopteran wasp *Nasonia* complex (Campbell *et al.* 1993). Most recently, Andreev *et al.* (1998) examined the ITS2 region of specimens of the coffee berry borer beetle *Hypothenemus hampei* from across the globe and found no variation in directly sequenced PCR products. The level of intra-individual variation was not addressed however.

Perhaps not surprisingly, the phenomenon of concerted evolution has been readily seized upon by researchers, since the apparent rapid homogenisation of the ITS regions suggests that it is not warranted, or indeed necessary, to sample many copies of an ITS sequence to deduce relationships between populations or species (Hillis and Dixon 1991). However, this may be folly. Studies covering a number of arthropods, for example two species of mosquito (Wesson *et al.* 1992; Tang *et al.* 1996); *Ixodes* spp. ticks, for both the ITS1 and ITS2 regions (Wesson *et al.* 1993; Rich *et al.* 1997), and tiger beetles (Vogler and Desalle 1994) have identified

significant intra-specific and even intra-individual variation in base composition. In the helminths, Hoste *et al.* (1995), report that two out of five species of *Trichostrongylus* spp. nematodes studied were found to have considerable intra-individual variability in their ITS2 sequences.

The presence of ITS variation, suggests that the homogenising effect of molecular drive may not be complete across all species, or even populations. Indeed, population aggregation analysis of rDNA sequence data carried out on the tiger-beetle, has shown the geographic pattern of the ITS1 distribution to be basically congruent with earlier mtDNA studies (Vogler and DeSalle 1994). Interestingly, when present, ITS variation is often in the form of variable numbers of simple sequence repeats (i.e. microsatellites; See Section 1.3.4.5), indicating a shared characteristic of variation.

Why seemingly species specific intra-individual rDNA variation occurs is at present a forum for conjecture. A number of authors have hypothesised that where intra-individual heterogeneity is present it may be as a consequence of the species in question being sub-divided historically into sub-populations or races, which are exchanging genetic material on the geographic "fringes" of their range. This may periodically introduce previously differentiated ITS sequences into a population, preventing homogenisation, and hence concerted evolution, across the species (see Tang *et al.* 1996; Vogler and DeSalle 1994).

That disparate populations have heterologous sequences is perhaps not a complete *non sequitur*. It would theoretically depend on the initial genetic variation

across populations and the time-scale necessary for the homogenisation of rDNA arrays to a single variant e.g. the phenomenon of concerted evolution could be applied to geographically isolated populations which may become homogenised for different variants, resulting in the geographic fixation of a particular sequence. Alternatively, a population moving towards homogenisation may have a mixed pool of transient inter- and/or intra-individual rDNA variants.

The research cited above has placed a fresh perspective on rDNA based studies, and as a consequence, it has become clear that multiple clones of separate ITS sequences *must* be analysed to examine any potential variation, both within an individual and between populations, before relationships between populations or species can be addressed with confidence. A PCR amplification product of an rDNA sequence will contain many amplicons (different amplification products) in the mixture, many of which will have been a separate amplification event from an individual rDNA array. If the sequences are truly homogenised then the consensus sequence identified directly from the PCR product may be correct, but there may also be a smaller proportion of other sequences overlooked. As a result, directly sequencing ITS regions from a PCR reaction, without cloning and screening multiple copies, could lead to a sequences being recorded which are not necessarily reflective of the true level of diversity present. This may in turn ultimately lead to an inaccurate conclusions being drawn regarding population or species relatedness. For example, Buckler *et al.* (1997) stress the need for identifying diversity in paralogous rDNA, whether it stems from multiple functional loci or from putative pseudogenes or recombinant molecules, to accurately assess relationships between organisms.

5.1.3. rDNA and spider studies

Many questions remain regarding the mechanisms and kinetics of concerted evolution. Only by examining organisms with different reproductive modes and dispersal capabilities will researchers be able to elucidate whether life history factors are in any way influencing molecular drive. The universality and accessibility of rDNA, and the questions the studies outlined above raise, makes the study of spider ITS regions a viable option in an attempt to gain insights into both the realms of population variation and, by an inter-specific comparison between the highly dispersive ballooning *L.tenuis* and the more sedentary *E.ovata*, into the possible effects that dispersal has on the variation within rDNA. It can be hypothesised that the more restricted dispersal, and hence restricted gene pool of *E.ovata* may lead to a greater level of between population variation than the highly dispersive *L.tenuis* i.e. each *E.ovata* population might homogenise towards a different rDNA variant if sufficient time has passed and a significant initial level of rDNA variation was present. For example, recent preliminary results from Navajas *et al.* (unpublished) have shown that two mite species, with strict ecological niches and restricted colonisation potential, revealed substantial geographical variation in the ITS2 region in comparison with a more ubiquitous and dispersive mite species. Admittedly, the comparison to spiders is initially confused by the reproductive system of mites, since they reproduce by arrhentoky (male haploidy) which tends to lead to lower genetic variation (since lethal mutations in haploid males select against these individuals in a population), but it is a workable framework on which to build the hypothesis.

This section of the project aims to assess genetic variation across a broad geographic range of *L.tenuis* and *E.ovata* populations via analysis of rDNA. In conjunction with the RAPD data (Chapter Four), results from a second DNA marker system may help to increase our understanding of how the different life history strategies of these spider species, with respect to dispersal, affects their levels of genetic variation. The rDNA analysis was conducted in two main stages.

The first was to PCR amplify, then digest with restriction enzymes, fragments of rDNA containing both the ITS1 and ITS2 regions, from a number of individual spiders from different locations. By separating the digests on polyacrylamide gels, any polymorphisms between populations which effected the restriction sequence would be visible. Variation, i.e. loss or gain of restriction sites due to point mutations or base substitutions, would then be reflected in the number of fragments generated. Positional changes in fragments are indicative of length polymorphisms caused by the insertion or deletion of bases. To achieve a greater degree of resolution, double digests were also carried out and the results examined on a higher percentage polyacrylamide gel.

The second stage of rDNA analysis was to clone and sequence a number of ITS1 copies from individuals from different locations, to gauge the level of intra-individual variation, in addition to examining inter-population variation at the sequence, the most definitive, level. The ITS1 region was chosen for further examination as it is reportedly generally more variable than the ITS2 (Schlotterer *et*

al. 1994). By assessing levels of sequence variation the extent to which concerted evolution has taken place could also be addressed.

In addition, the consensus ITS1 regions of each species were folded into hypothetical secondary structures to generate additional information about species relatedness and intra-specific variation. Furthermore, as the study generated sequence data on spider 5.8S and 28S genes, these were applied to phylogenetic analyses to assess their potential for resolving systematic issues within the Arachnida.

5.2. MATERIALS AND METHODS

5.2.1. PCR amplification of rDNA

PCR reaction conditions and primer designations were largely as reported by Fenton *et al.* (1997), successfully used for the study of Acarid rDNA (see Section 2.6.7.2 for optimised conditions). Minor alterations in the cycling parameters (reduction in annealing time and removal of ramp rate) were required to reduce smearing of the PCR product. Such alterations are often necessary when utilising a different thermal cycler, due to the slight inconsistencies in heating and cooling capabilities between PCR machines. The Mg^{2+} concentration was also reduced to 1.5mM.

Three sets of primers were used to amplify genomic spider rDNA. Primers C and E were used to amplify a fragment containing both the ITS1 and ITS2 regions and the 5.8S gene; primers C and B the ITS1 region; and primers G and E the ITS2 region (see Table 5.1 for primer sequences and binding sites).

Table 5.1. PCR Primers and their annealing position used to amplify spider rDNA. Primer length is in parenthesis.

Primer	Annealing Location	Sequence (all 5'-3')	Length
Primer C	conserved 18S gene	AGAGGAAGTAAAAGTCGTAACAAG	(24nt)
Primer B	conserved 5.8S gene	GCTGCGGTCTTCATCGATCC	(20 nt)
Primer G	complement of B	GGATCGATGAAGACCGCAGC	(20 nt)
Primer E	conserved 28S gene	CAACTTTCCCTCACGGTACTTG	(22 nt)

5.2.2. PCR product concentration and clean-up

Re-precipitation of the PCR product was carried out both to concentrate the amplified product prior to digestion with restriction enzymes, and also to remove some of the smaller fragments and residual dNTPs remaining in the PCR mix (Sambrook *et al*, 1989). If the amplification was successful (i.e. a single product of expected size when viewed on a gel) the remaining PCR reaction was pipetted into a fresh tube. One-tenth volume 2 M sodium acetate and 2.5 volumes of absolute alcohol were added, mixed gently by inversion, then placed at -20 ^{o}C for a minimum of 1 hour to allow precipitation of the DNA. The tube was then spun at 12,000 rpm for 10 minutes to pellet the re-precipitated DNA. The pellet was washed with 70 % alcohol and allowed to air dry. Finally, the pellet was resuspended in 20 µl SDW.

5.2.3. Restriction digests of PCR fragments

Ten microlitres of cleaned-up and resuspended PCR product were digested in the presence of 1x One-Phor-All Buffer (Pharmacia, UK) with 2-3 units of restriction enzyme. Between five and 12 individuals of each species from various sites were analysed. Digests were placed in a heating block for a minimum of one hour at the appropriate optimal enzyme activity temperature.

Following incubation, the tubes were pulse spun at 12,000 rpm to recover any evaporate on the lids of the tubes, and 3 µl loading buffer added. Size separation of digested fragments was then conducted on a 10 % non-denaturing polyacrylamide gel. Double digests were carried out as above but with 2-3 units of each enzyme. In

the case of *Dde*I/*Taq*I double digests, which required two different temperature regimes, a PCR machine was programmed to heat at 37 °C for two hours followed by 65 °C for two hours (the lower temperature was conducted first to prevent denaturing the less hydrothermic enzyme). The restriction fragments were then analysed on a 16 % polyacrylamide gel. A higher resolution gel was used for double digests since the fragments can be anticipated as being of a smaller size. The following enzyme/enzyme combinations were used: *Eco*RI (6 base cutter), *Dde*I (5 base cutter), *Hae*III (4 base cutter), *Taq*I (4 base cutter), *Dde*I/*Taq*I and *Hae*III/*Eco*RI.

5.2.4. Cloning of PCR products

Two cloning kits were employed during the study - the Stratagene pkBluescript cloning kit, and the Invitrogen Blunt-PCR kit. Both kits contained a vector that ligates blunt ended PCR products - which *Taq* polymerase does not generate (one of the peculiarities of *Taq* polymerase is that it adds an adenine residue overhang onto the 3' end of the amplified fragment). A number of other polymerases, e.g. *Vent* polymerase, do produce blunt ended fragments, but are considerably more expensive than *Taq*. Fortunately it is relatively simple to generate blunt PCR fragments by using T4 DNA polymerase.

5.2.4.1. Blunt ending PCR products for ligation

The blunt ending reaction mix comprised of the following: 10 µl re-precipitated PCR product, 1 µl 10x One-Phor-All buffer (Pharmacia), 0.5 µl T4 DNA polymerase and 0.5 µl dNTPs (10 µM). This was incubated at 12 ^{o}C for 12 minutes (creating an ice/water bath in a polystyrene box was the most effective method of achieving this temperature), then the T4 polymerase denatured by heating at 70 ^{o}C for a further 10 minutes on a heating block. The T4 polymerase incorporates dNTPs onto the 3' end of the DNA fragment, filling in and removing any overhangs, thus producing a blunt PCR fragment.

5.2.4.2. PCR fragment ligation

Both cloning kits utilised a different vector system designed to prevent vector-to-vector re-ligation during the ligation process. In the case of the Stratagene kit, re-precipitated, blunted PCR product was ligated at 12 ^{o}C for 4 hours. The following components were added in a total reaction volume of 10 µl: 6.5 µl PCR product, 1 µl ligase, 1 µl plasmid vector, 1 µl 10x ligation buffer and 0.5 µl *Srf*I restriction enzyme. The ligation buffer contains ATP which degrades after multiple freeze thaw cycles, and so as a precaution 1 µl of additional ATP was added to ensure the ligation reaction had sufficient energy. The restriction enzyme *Srf*I prevents the vector re-ligating to itself by cleaving at the restriction site created if the vector re-anneals, thereby limiting false positives. The ligation was stopped by denaturing the enzyme at 70 ^{o}C for 10 minutes.

Invitrogen's PCR-Blunt cloning kit, in contrast, relies on the formation of a lethal gene if vector to vector re-ligation occurs, thereby ensuring only bacteria with vector containing a fragment can survive following plating. The ligation reaction was carried out for one hour at 16 ^{o}C as described above, although the *Srf*I was not required, and with the recommended 5 µl blunted PCR product (2 µl SDW was added to increase the total reaction volume to 10 µl).

5.2.4.3. Vector transformation

Both vectors were immediately transformed into supercompetent bacterial cells using a heat-shock method following the respective manufacturers' instructions (this involves heating pre-cooled cells, allowing the plasmid vector to penetrate the cell membrane, then placing the cells on ice to recover).

The cells were allowed to recover in Luria-Bertani broth (LB; Bertani 1952) medium for one hour at 37 ^{o}C with shaking. Care was taken to ensure recovery time did not exceed the one hour recommended by the manufacturer. This ensures that the majority of clones have resulted from independent ligation and transformation reactions and have not undergone cell division (which could bias the proportion of a certain sequence in the transformation mix).

5.2.4.4. Plating and selection

The transformed cells were plated under sterile conditions in a laminar flow cabinet onto LB agar plates containing an appropriate antibiotic for the selection of

the transformed bacteria: Ampicillin (25 μg/ml) for the Stratagene kit and kanamycin (50 μg/ml) for the Invitrogen kit, then left overnight at 37 ^{o}C.

Colonies which were visible following incubation (indicating they should contain a plasmid which has ligated with a PCR fragment) were carefully picked off with a pipette tip, and grown overnight in 3 ml LB (again containing the appropriate antibiotic) in preparation for a plasmid mini-preparation.

5.2.5. Plasmid DNA preparation and subsequent plasmid digests

Plasmid DNA boil preparations (often referred to as mini-preps) (Sambrook *et al.* 1989) were executed to assess if an appropriate sized insert was present. Before carrying out the procedure however, it is vital to sub-culture each putative positive, to enable the generation of more material should further analysis be required. This is simply done by dipping a pipette tip into the overnight culture and placing it in fresh medium.

Samples were centrifuged at 14,000 rpm in a microfuge for two minutes to pellet the bacterial cells and the supernatant (the liquid medium) decanted. Three hundred and fifty microlitres STET (0.1M sodium chloride, 10mM Tris-HCl pH8, 1mM EDTA and 5% Triton X-100) and 25 μl lysozyme (10mM Tris + 100mg lysozyme) were added to each tube, and the tubes vortexed to resuspend the cells. The tubes were placed in boiling water for exactly 40 seconds to lyse the cells then spun at 12,000 rpm for 10 minutes. The STET precipitates the bacterial debris whilst leaving the plasmids free in the supernatant. A toothpick was used to remove the

bacterial waste. This waste material can be quite viscous, and care must be taken to ensure it is removed without shearing and falling back into the supernatant. Any bacterial waste left behind may contaminate the plasmid preparation and hinder future manipulations.

Plasmid DNA was then precipitated by the addition of 40 µl 3 M sodium acetate and 420 µl isopropanol to the remaining supernatant. The tubes were briefly vortexed, left at room temperature for five minutes, spun at 12,000 rpm for 5 minutes and then the isopropanol decanted. The pellet was washed with 70 % ethanol then allowed to air dry. Finally, the plasmid pellet was resuspended in 20 µl TE containing RNase A (Boehringer-Mannheim, UK) (1 µl per 400 µl T.E.) and incubated for two hours at 37 ^{o}C. The RNase step is necessary to remove the very large amount of RNA released from the bacterial cells during lysis.

Ten microlitres of the resuspended plasmid was then digested for two hours at 37 ^{o}C with the appropriate restriction enzyme(s) (3 units/sample) to release the plasmid insert (*Sma*I and *Hin*dIII for the Stratagene kit and *Eco*RI for the Invitrogen kit). The products of the digest were run out on a 1.4 % agarose gel and stained with ethidium bromide. A band present at the expected size gave initial confirmation. However, the sample was then verified via PCR using the primers which originally amplified from the genomic DNA (see Figure 5.2 for the primer binding sites).

5.2.6. Checking putative positive inserts and preparing plasmids for sequencing

A 1/100 SDW dilution of plasmid DNA, containing a putative rDNA insert, was used as template for a PCR reaction using the primers and conditions as used initially for amplification. However, a shorter 25 cycle programme was employed because of the purity and high copy number of the sample. Following amplification, an aliquot of the PCR product was electrophoresed on an agarose gel, stained with EtBr and examined under UV illumination. If a product of expected size was present, a 3 ml bacterial culture (initiated using the sub-culture prior to mini-prepping) was grown overnight in LB broth with the appropriate antibiotic at 37 ^{o}C, then the plasmids prepared for sequencing with a Wizard miniprep kit (Promega, UK), following the manufacturer's instructions. The kit produces a higher quality "cleaner" plasmid preparation which is more suitable for sequencing (a purer plasmid template reduces the chance of mis-priming of the sequencing primers) than the faster boil preparation. The kit is not used routinely to check putative positives however, because of the prohibitive cost.

5.2.7. Sequencing

The plasmid inserts were sequenced on an ABI Prism 377 automatic sequencer operated by Oswel DNA Services, Southampton, UK. The initial sequences were generated using the M13 "universal" forward and reverse primers which anneal to sites present on the cloning vector, then, having confirmed the sequences were the correct putative region, the original PCR primers were subsequently used (Figure 5.2). A single full length clone of *E.ovata* which covered

the ITS1, ITS2 and included the 5.8S gene was sequenced in both directions. Unfortunately there were repeated problems with primer binding with the cloned *L.tenuis* fragments and a full length read through the 5.8S gene was not possible for this species.

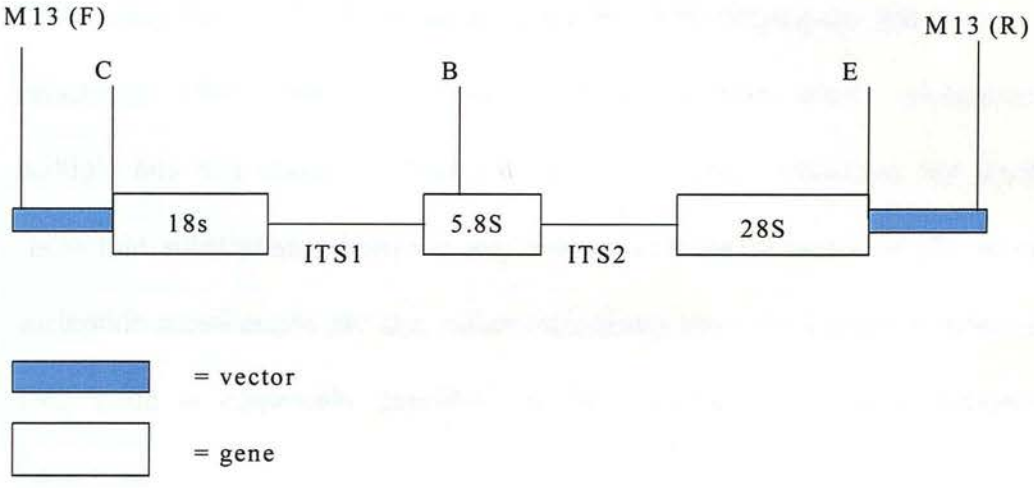


Figure 5.2. Diagram of a cloned rDNA array and surrounding vector. The primer binding sites are indicated (C, B and E ribosomal primers; M13(F) and M13(R) are vector primer sites.

5.2.7.1. Analysis of ITS1 regions

Multiple ITS1 clones (2-4) from a single individual at each location were sequenced i.e. from Scotland, England and New Zealand for *L.tenuis* and from Elgin, Edinburgh and Invergowrie for *E.ovata*. Moreover, a single individual of *Bathyphantes gracilis* (Blackwall), a Linyphiid with a less dispersive nature than *L.tenuis*, from East Lothian, Scotland, was also amplified with primers C and B

(ITS1 region) and the product sequenced. The ITS1 clones were all sequenced in one direction only with primer C from PCR fragments generated with primers C and B.

The ITS1 sequences within each species were aligned with CLUSTAL W 1.6 (Thompson *et al.* 1994), using the default parameters, and the degree of variation between sequences indexed via the Kimura two-parameter distance measure (Kimura 1980) using the DNADIST program of the PHYLIP (Phylogeny inference package; Felsenstein 1993). The Kimura two-parameter method, which compensates for multiple hits and biases in transition/transversion rates, calculates the number of nucleotide substitutions between sequences expressed in terms of the number of nucleotide substitutions per site, rather than simply the total number of substitutions. This value is commonly described as the evolutionary distance between two sequences.

The consensus ITS1 sequences from *B.gracilis*, *L.tenuis* and *E.ovata* were also aligned with each other using CLUSTAL W 1.6 (default settings) and compared via the Kimura two-parameter distance measure, to assess the degree of ITS1 similarity between the species (Section 5.3.4.7). In addition, to gain further information on variation and species relatedness the minimal energy hypothetical secondary structure of the ITS1 regions of each species was constructed using "Mfold" (Zucker 1989) (See section 5.3.4.8).

5.2.7.2. Phylogenetic analysis

Initially, the 5.8S gene and 28S gene fragments of *E.ovata* were compared to those available in the NCBI (National Centre for Biotechnology Information) sequence database, to identify and verify their origin. The PHYLIP programs SEQBOOT, DNAPARS, DNADIST, NEIGHBOR and CONSENSE were then used to create phylogenetic trees via two different dendrogram building methods.

The *E.ovata* 5.8S gene sequence (108 bases) was compared to the 5.8S region of a number of invertebrate representatives (two insects, three mites, a tick and a mollusc). Suitable sequences for comparison were accessed from the NCBI GenBank sequence database, and aligned with the *E.ovata* sequence via Clustal W 1.6 using the default parameters. The mollusc was used as an outgroup. A similar process was repeated with the 28S sequence data (some 300 bases in length) and dendrograms generated from *E.ovata* and *L.tenuis* with homologous regions from three mite species, a tick and the spider *Eurypelma californica*, with *Drosophila melanogaster* acting as the outgroup. This would allow a closer analysis of the taxonomic relationships within the Arachnida.

Firstly, 500 bootstrap data sets were created (SEQBOOT; to create pseudo replicates of the data) and from these, genetic distance matrices (DNADIST) were generated based on the degree of consensus sequence variation (Kimura two-parameter distance measure) and used to produce trees via the UPGMA option in the NEIGHBOR program. The CONSENSE program then examined the 500 trees and presented the tree with the most common topology. A maximum parsimony tree (DNAPARS) was also created from the bootstrapped data.

5.3. RESULTS AND DISCUSSION

5.3.1. PCR Results

The conserved primers proved highly effective with spider genomic DNA. All three primer pairs successfully amplified single fragment products with the species examined.

Table 5.2. Size of PCR fragments approximated from agarose gel

Species	Primer pair	Region amplified	Size (bp)
L.tenuis	C+E	ITS1 + 5.8S + ITS2	1200
E.ovata	C+E	ITS1 + 5.8S + ITS2	1300
B.gracilis	C+E	ITS1 + 5.8S + ITS2	1100
L.tenuis	C+B	ITS1	450
E.ovata	C+B	ITS1	500
B.gracilis	C+B	ITS1	400
L.tenuis	G+E	ITS2 + 28S	750
E.ovata	G+E	ITS2 + 28S	800

Logically, the fragment amplified with primers G and E (containing the ITS2 region and a portion of the 18S gene) should, when summed with the fragment amplified with primers C and B, make a product the size of C and E (See Figure 5.2). This was indeed the case.

The fragment size estimates (Table 5.2), although rough, were comparable with a number of other arthropod studies. *Cincindela dorsalis*, the Tiger beetle, has the smallest reported insect ITS1 region at 255-268 bp (Vogler and DeSalle 1994),

whilst *Drosophila* spp. have some of the largest recorded at 529-851 bp (Schlotterer *et al.* 1994). The mosquito *Aedes aegypti*, and *Cecidophyopsis* spp. mites, have ITS1 regions of 419 bp (Wesson *et al.* 1992) and 357-386 bp (Fenton *et al.* 1997) respectively.

5.3.2. PCR-RFLP analysis of the rDNA array

The primary advantages of restriction site analyses over sequence based analysis are that several individuals can be assessed quickly and cost effectively. The main disadvantage of the technique is the biased nature of the analysis (wholly dependent on the restriction enzymes chosen) and the lower level of detail which can be gathered.

5.3.2.1. Single digests

No obvious restriction site loss or gain were identified between populations within either *L.tenuis* or *E.ovata*, with any of the restriction enzymes used. Indeed, *Eco*RI (a 6 base cutter) did not restrict the rDNA fragment in either species. This was ultimately fortuitous, since the Invitrogen cloning kit employs this enzyme to release the vector insert. This allowed the restriction of the vector to act as a test of the insert, since if it was restricted, and two (or more) bands appeared it could not be the correct fragment and could be excluded from further analysis. However, despite the lack of restriction site differences, there was an indication of length polymorphisms in a small number of samples, meriting further investigation with a double-digest which

would act to increase the resolution of the assay (whilst maintaining the ability to screen a number of samples rapidly).

5.3.2.2. Double digests

The double digests did not satisfy in terms of the generation of obvious restriction site polymorphisms, as the samples from all locations produced an identical number of fragments. However, there was once again an indication of length polymorphisms in a few samples (Figure 5.3). These may have been due to the presence of SSRs in the ITS regions, as reported by several other authors. Sequencing of the cloned fragments would allow analysis of these regions at the highest possible resolution.

Overall, the RFLP analysis of the rDNA array shows comparable i.e. very low levels of variation present in rDNA arrays of both *E.ovata* and *L.tenuis* between the sampled populations. Only by sequencing however, could intra-individual variation be assessed and questions about any effect of dispersal on the phenomenon of concerted evolution posed. In retrospect, once the sequence data for the *E.ovata* full-length clone had been determined, it was possible to map the restriction sites of the enzymes used in the study, via the Cutter on-line package (Heinman 1995; http://www.medkem.gu.se/cutter/), and compare the RFLP fragments and the actual sequence data. This allowed verification that the restriction patterns produced on the gel were accurate.

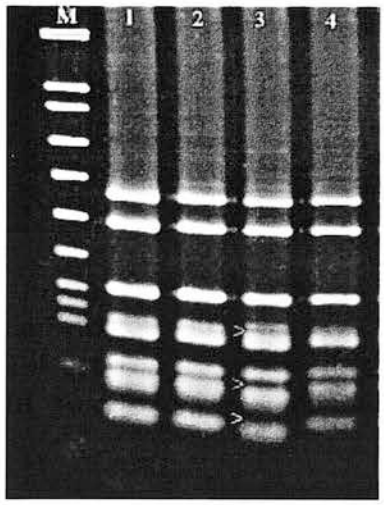


Figure 5.3. Double digest of PCR amplified rDNA with *DdeI*/*TaqI* restriction enzymes.
Lanes 1-4 are *Enoplognatha ovata* individuals from Invergowrie. The arrows highlight possible length polymorphisms in lane three. M = molecular size marker.

5.3.3 Cloning results and troubleshooting

Cloning the *E.ovata* rDNA fragment (encompassing both ITS1 and ITS2) utilising the Stratagene cloning kit was successful, but only after a number of failed attempts. The greatest problem seemed to arise as a result of ligating the re-precipitated PCR product directly into the vector without prior gel-excision and purification. Foregoing this step led to a number of products of approximately 500 bp consistently being ligated in preference to the desired 1300 bp product. Presumably, despite the agarose gel indicating that only one product was present, a few copies of the smaller fragment (either partial amplifications or perhaps even from the initial genomic template itself) were also present. The smaller size of the fragment would make it more amenable to ligation. After several early attempts at cloning, a 500 bp product was present as an insert with such consistency that it was postulated that

perhaps the PCR product was being cleaved by the enzymes used to release the insert from the vector (*Hin*dIII and *Sac*I), leading to small fragments being present on the agarose gel. However, the PCR product itself was not digested with these enzymes when tested directly, so this hypothesis was rejected. If resources were not limiting, the fragment could have been sequenced to identify accurately its origin. However, success was eventually achieved by screening a large number of putative positive colonies.

The ITS1 region from *L.tenuis*, *E.ovata* and *B.gracilis* was cloned with the Invitrogen kit, which proved far more effective in cloning. This may in part be due to the smaller size of the ligated fragment but perhaps also due to the superior system of preventing vector-to-vector re-ligation due to the formation of a lethal gene. In practical terms, the critical factor seemed to be ensuring the PCR product was highly concentrated prior to ligation. Forty microlitres of PCR product was re-precipitated and re-suspended in 10 µl SDW. Of this, 5 µl was used in the ligation reaction (the maximum volume recommended in the kit guidelines), and this produced a 40-45 % success rate (i.e. colonies actually containing an insert). Using a less concentrated PCR product resulted in very few transformants which did not appear to contain inserts. The plasmid preparation and subsequent PCR screening (Sections 5.2.5 and 5.2.6) proved fast and highly effective methods of identifying the cloned inserts.

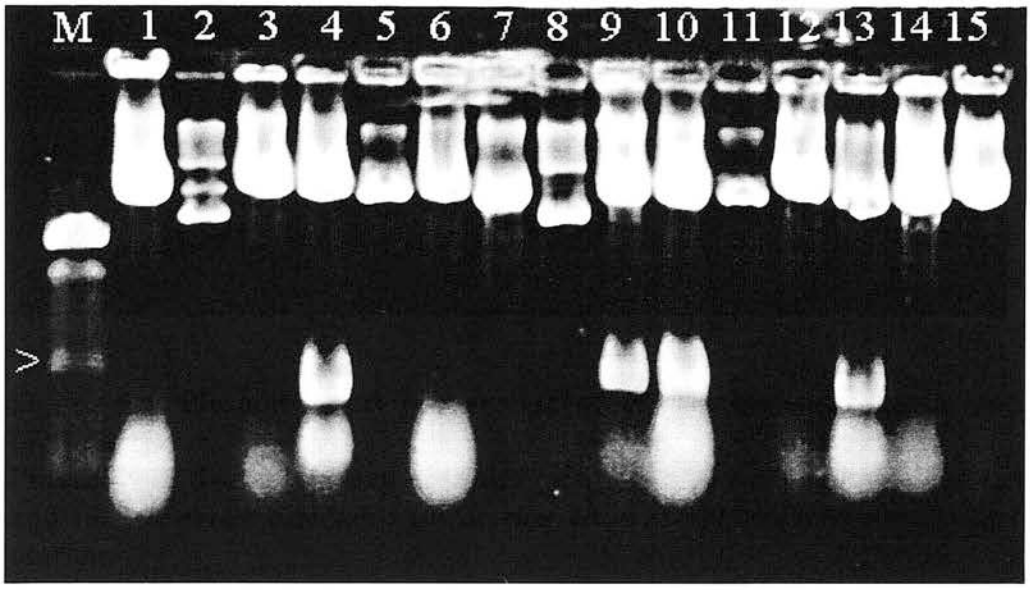


Figure 5.4. Plasmid digest preparation of 15 *Lepthyphantes tenuis* putative ITS1 clones.
Putative positives of the expected size (450 bases) are present in lanes 4, 9, 10 and 13. The arrow highlights the marker band at 600 bases. M = molecular size marker.

5.3.4. Sequence data

5.3.4.1 *E.ovata* rDNA array

Data for an entire rDNA array was obtained for one *E.ovata* clone from Invergowrie, Dundee (Figure 5.5). Where base ambiguities in the sequence were recorded by the automatic sequencer, a visual examination of the peaks on the sequencer readout (chromatograph) allowed a correction to be made. The putative position of the 5.8S gene (108 bases) is underlined centrally and contains the primer site B (the complement of which is primer G). The position of the 5.8S gene boundaries have been chosen by reference to other invertebrate sequences for the same region. Sixty bases of the 18S gene and 365 bases of the 28S gene are also

highlighted by underlining. The sequence has been deposited in the NCBI GenBank using the BankIt program and has accession number AF084942.

18S gene

primer C

5'**GAGGAAGTAAAAGTCGTAACAAG**GTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGATGGTTG
TGTATTGGCTCAACACCATCACCTTGACACGGGGTGCCCGAGAAGGTTCTCCCGCAACTCTGGCGTAT
CCGCTCACGGTTCGCGAAGAGTGACAACAATTCGAACGCAACTTTGAACGCTGAAGCGTCTCGGTACC
TCATGGGTGTGCCTATTTATTCCCCGGCCAACAACATGGCAAGTGGGAGGTCTCTTTTGAGGCTAGGC
TTGAAGAGGGGGTCTCACCGACCTCGCCGCCCCGTTGGTGCCTTGACCCGGTGGGGGTCTCTGGAGTA
TTAGTCCCGGGGACTATCGGACTTGGAAATCTTGTCCGTCTCTGGAGTGCCCTTTCCGTGAACAAGAC
TCATCCCCGGACCTTCCTAACGGAAGAAATCCTAAATTTTATAAAATCTCGAACGCTAATAAGGCTTC
CGTGAGGTTGCCGAAACTTGACAACTCTGAGCAGTGGATCACTCGGCTCACG**GGTCGATGAAGAACGC**
AGCCAGCTGCGAGACTGGTGTGAATTGCAGGACACATTGAGCACTGATTTTTCGAACGCGCATTGCGG
CCTCGGGTCCTGCCCGGGGCCTCGCCTGTCTGAGGGTCGGATAAGACTTGCAAAGGAAAGTTCGCTTT
CCACTTGGCCGAACCGGATCGCTTTTGTGGTTCGCCGGCTTAAGGTTTACGGATCCTCCCCGGCCGAG
AAGCGTGGCTCCCACTTGACCAACGCTCGCGGGCTGGAGAAGACTGAGTAGTTTCTCCGCTGAAGCGA
GCGACGGGAGCGAGCGCAGAGAGCTTGCCGAGGAGAGATCCTCCACGCGTGCCACTGGCACGGTAAAC
TCAAAAATATTGTCGACCTCAGATCAGACGAGATGACCCGCTGAATTTAAGCATATAAATAAGCGGAG
GAAAAGAAACCAACAGGGATTCCCTGAGTAACGGCGAGCGAAAAGGGAAGAGCCCAGCGCCGAATCCC
GAGCCTTTTGGGTTTCTGGGAAATGTGGCGTTTAGGAGTGAGCGTGCCGGAGGTTGACTCTGCAGCAA
GTCCCCCTGACCGGGGCTTTTATCCAGAGCGGGTGCCGAGGCCCATAGCTGCTAGGTCTCCTTCGCAT
GTGATCTCCTTGGAGACGGGTTGCTAGGGAGTGCAATCCTAATCGGGTGGTAAACTCCACCTAAGGGT
AAATACTGCCGTGAGACCGATAGCAAA**CAAGTACCGTGAGGGAAAGTTG**3'

28S gene

primer E

Figure 5.5. ***Enoplognatha ovata*** **rDNA array.**
The primer sequences are in bold, and the conserved genes underlined. The putative position of the 5.8S gene (108 bases) is underlined centrally. The overall length is 1271 bases.

Interestingly, there is a degree of mis-match between the 5.8S primer (primer B) used in the amplification of the ITS1 region; 5'-GGATCGATGAAGACCGCAGC-3' and the actual *E.ovata* sequence; 5'-GGTCGATGAAGAACGCAGC-3'. The first adenine in the primer sequence is absent from the spider, and similarly, an additional adenine in the spider sequence is present as a cytosine in the primer. The second observation (the adenine to cytosine substitution) is also found to occur in several mite species (Fenton *et al.* 1997), perhaps highlighting the mutation as an arachnid characteristic. Single internal mis-matches are thought to have little effect on PCR product yield

when the primers are relatively large if there are a number of matched bases on either side of the mismatch (Palumbi 1996). That would appear to be the case here. In the event of further rDNA based studies however, a spider specific primer could be synthesised using the actual sequences, which may allow a higher annealing temperature to be set increasing the stringency of the reaction. The 5.8S sequence was generated from a PCR product originally amplified with primers C and E. Notably, the sequenced ITS1 regions were all amplified using primers C and B, so as a result the primer sequence rather than what can now be considered the actual *E.ovata* sequence is present in the data. The *L.tenuis* 5.8S gene could not be compared, because as stated a complete sequence was not achieved.

5.3.4.2. ITS1 sequence analysis

5.3.4.3. General description of spider ITS1 regions

The cloned ITS1 regions of *L.tenuis* were identical in length, both between populations and within individuals, at 394 bases. The *E.ovata* ITS1 sequences were larger, ranging from 444-448 bases. The *B.gracilis* ITS1 region was the shortest of the three species, with the cloned sequences ranging from 339-341 bases. The alignment of sequences within each species was unambiguous due to their near complete homology. As above, bases which were ambiguously assigned by the automated sequencer software were examined by eye and amended if appropriate. Similarly, due to the low level of variation, the bases which were identified as polymorphic from the consensus sequence were also ratified as being accurate by referring back to the original chromatograph. The *L.tenuis* and *B.gracilis* consensus

ITS1 sequences were submitted to the NCBI GenBank via the BankIt program with accession numbers AF086784 and AF086785 respectively.

Short tandem repeats were present in the ITS1 region of all three spider species but as indicated by the low level of overall length variation, there was no difference in the number of the repeats in any of the clones within a species (Table 5.3). The repeats are not necessarily distributed independently of each other i.e. the sequence TCTCTA was recorded as two repeats - TCTC and CTCT.

Table 5.3. Summary of di- and tri- nucleotide repeats found within the consensus ITS1 regions of *Enoplognatha ovata*, *Lepthyphantes tenuis* and *Bathyphantes gracilis*. These short sequence repeats (SSRs) were constant in their position between clones and did not show variation in motif length. The position of the repeat (Pos.) refers to the sequences presented in Sections 5.3.4.4, 5.3.4.5 and 5.3.4.6.

E.ovata	No.	Pos.	*L.tenuis*	No.	Pos.	*B.gracilis*	No.	Pos.
AAAA	1	431	TTTT	1	44	CCCC	1	64
TTTT	2	239,425	CCCC	1	248	GGGG	3	80,224, 228
GGGG	5	76,260,305, 306,329	GGGG	4	271,272, 286,402	CTCT	1	357
						GAGA	3	90,97, 239
CCCC	1	282,	CTCT	1	429	CGCG	1	111
AGAG	1	134	TCTC	2	80,338	CACA	1	158
GAGA	1	85	CACA	1	414	TCTC	1	58
TGTG	2	44,192	GTGT	3	117,230,351	TGTG	1	165
GTGT	1	191	TGTG	2	116,231	GTGT	1	294
CTCT	5	106,236,311, 360,481	GCGC	3	145,306,406	TTTTTT	1	282
			CGCG	2	144,255	CTCTCT	1	57
TCTC	5	175,235,265, 311,359	AGAG	1	299	GGAGGGAG	1	203
			GAGA	1	301	CAAACAAA	1	305
CGCG	1	128	TATA	1	380	TCCCTTCCCT	1	275
TATA	1	429	CGACGA	1	384			
TAATAA	1	445	GCTTTGCTTT	1	172			
GAAGAA	1	411						
CAACAA	2	141,214						
GCCGCC	1	278						
TATTTATT	1	198						

The base composition of the consensus (most common) sequences of each cloned spider ITS1 region (Table 5.4), is comparable with a number of other arthropod studies e.g. *Anopheles* mosquito ITS1 contained 58 % GC (Wesson 1992), *Cecidophyopsis* mites 40 % (Fenton *et al.* 1997) and *Ixodes* ticks were found to contain 49 % (Wesson *et al.* 1993). However, these results are in contrast to *Cincindela dorsalis* which has an unusually low (21 %) GC content (Vogler and DeSalle 1994) and the study of Schlotterer *et al.* (1994) which reported the *Drosophila* spp. GC content < 30 %. A low GC content is generally not typical for ITS spacers (reviewed in Torres *et al.* 1990). The ITS2 region of *E.ovata* was 293 bases in length and contained 59 % GC content (very similar to the 56 % recorded in the mosquito *Anopheles nuneztovari* (Fritz *et al.* 1994)). This slightly increased GC content in the *E.ovata* ITS2 region relative to the ITS1 is, however, in contrast to that reported generally in mosquitoes (Wesson *et al.* 1992). The significance of GC content in the ITS region is not entirely clear, but it has been suggested that in coding regions, elevated GC content is linked to a move to warm bloodedness in animals, and in a move from arid to temperate regions in plants. Wesson *et al.* (1992) postulate that the relatively high GC in mosquito's may be associated with their warm-blooded feeding habits. This can obviously be discounted for spiders. The possibility of climate affecting the GC content could easily be determined by examining samples of spiders from more localities with diverse climates.

Table 5.4. Base composition of the ITS1 region of *Lepthyphantes tenuis*, *Enoplognatha ovata* and *Bathyphantes gracilis*.

Species	% GC content	% AT content	% A	% T	% C	% G
L.tenuis	56	44	20	24	27	28
E.ovata	53	47	22	25	26	27
B.gracilis	55	45	22	23	27	28

5.3.4.4. *Enoplognatha ovata* ITS1

The consensus length of the *E.ovata* ITS1 region was 445 bases. Intra-specific ITS1 alignments show a single clone from Elgin contained a three base insertion (CGT) which was absent in all other clones, and one sequence from Invergowrie contained a single base deletion. Assessing the total number of substitutions and deletions/insertions, 12 mutation events (shown in bold in Figure 5.6) were present across the ITS1 region of the 10 *E.ovata* sequences. When assessing the number of mutation events, it has been assumed that contiguous insertions and deletions, relative to one another, represent a single event. However, contiguous base changes, transitions (A $\leftrightarrow$ G or C $\leftrightarrow$ T) and/or transversions (A or G $\leftrightarrow$ C or T), are assumed to represent independent events. Notably, each polymorphism was unique to a single clone. Thus, in total some 2.7 % (or 12/445 sites) of the *E.ovata* ITS1 sequence was variable. The average number of nucleotide substitutions per site, indexed via Kimura's two-parameter estimator, was 0.0038. Of the 10 substitution events, six were transitions and four transversions. There is an indication of the presence of mutational "hot spots", as reported by other authors e.g. McLain *et al.* (1995), in two regions. Firstly, between bases 140-158 there are three

polymorphisms, and again between bases 422-429 where three polymorphisms are also found. These hot spots are thought to be indicative of areas of particularly low selection pressure which probably do not affect the folding of the secondary structure following transcription and so undergo relatively greater mutation rates. A method of assessing if this is the case is to model the possible secondary structure of the ITS1 region and map where the mutations are occurring and examine how this affects the overall structure. This has been described for the three spider ITS1 regions in Section 5.3.4.8.

Notably, six of the 12 *E.ovata* mutation sites are preceded by an adenine base. Particularly interesting is that two of the substitutions to adenine occur in the sequence position immediately after a CAA di-repeat, as these repeats are more prone to replication error both *in vivo* and *in vitro* (Schlotterer and Tautz 1991). For example, this type of SSR was found to be present in eriophyid mites and length differences caused by the number of these repeats was a major cause of inter-specific variation (Fenton *et al.* 1997).

Figure 5.6. Multiple alignment of the cloned ITS1 sequences from *Enoplognatha ovata*. Inv = Invergowrie, Elg = Elgin, Ed = Edinburgh. The number refers to each clone. Bold represents a substitution, - represents a deletion or gap. An asterisk highlights a site of mutation. The positions of the ITS1 region, 18S and 5.8S gene are indicated.

```
                                                  18S gene < > ITS1
                1                                                 45
1 Inv4          AGGTTTCCGTAGGTG AACCTGCGGAAGGAT CATTACCGA TGGTTG
2 Elg2          AGGTTTCCGTAGGTG AACCTGCGGAAGGAT CATTACCGA TGGTTG
3 Elg1          AGGTTTCCGTAGGTG AACCTGCGGAAGGAT CATTACCGA TGGTTG
4 Ed2           AGGTTTCCGTAGGTG AACCTGCGGAAGGAT CATTACCGA TGGTTG
5 Ed1           AGGTTTCCGTAGGTG AACCTGCGGAAGGAT CATTACCGA TGGTTG
6 Ed3           AGGTTTCCGTAGGTG AACCTGCGGAAGGAT CATTACCGA TGGTTG
7 Inv3          AGGTTTCCGTAGGTG AACCTGCGGAAGGAT CATTACCGA TGGTTG
8 Ed4           AGGTTTCCGTAGGTG AACCTGCGGAAGGAT CATTACCGA TGGTTG
9 Inv2          AGGTTTCCGTAGGTG AACCTGCGGAAGGAT CATTACCGA TGGTTG
10Inv1          AGGTTTCCGTAGGTG AACCTGCGGAAGGAT CATTACCGA TGGTTG

               46                                             *  90
1 Inv4          TGTATTGGCTCAACA CCATCACCTTGACAC GGGGTGCCTGAGAAG
2 Elg2          TGTATTGGCTCAACA CCATCACCTTGACAC GGGGTGCCTGAAAAG
3 Elg1          TGTATTGGCTCAACA CCATCACCTTGACAC GGGGTGCCTGAGAAG
4 Ed2           TGTATTGGCTCAACA CCATCACCTTGACAC GGGGTGCCTGAGAAG
5 Ed1           TGTATTGGCTCAACA CCATCACCTTGACAC GGGGTGCCTGAGAAG
6 Ed3           TGTATTGGCTCAACA CCATCACCTTGACAC GGGGTGCCTGAGAAG
7 Inv3          TGTATTGGCTCAACA CCATCACCTTGACAC GGGGTGCCTGAGAAG
8 Ed4           TGTATTGGCTCAACA CCATCACCTTGACAC GGGGTGCCTGAGAAG
9 Inv2          TGTATTGGCTCAACA CCATCACCTTGACAC GGGGTGCCTGAGAAG
10Inv1          TGTATTGGCTCAACA CCATCACCTTGACAC GGGGTGCCTGAGAAG

               91         *                                  135
1 Inv4          GTTCTCCCGC---AACTCTGGCGTATCCGC TCACGGTTCGCGAAG
2 Elg2          GTTCTCCCGCCGTAACTCTGGCGTATCCGC TCACGGTTCGCGAAG
3 Elg1          GTTCTCCCGC---AACTCTGGCGTATCCGC TCACGGTTCGCGAAG
4 Ed2           GTTCTCCCGC---AACTCTGGCGTATCCGC TCACGGTTCGCGAAG
5 Ed1           GTTCTCCCGC---AACTCTGGCGTATCCGC TCACGGTTCGCGAAG
6 Ed3           GTTCTCCCGC---AACTCTGGCGTATCCGC TCACGGTTCGCGAAG
7 Inv3          GTTCTCCCGC---AACTCTGGCGTATCCGC TCACGGTTCGCGAAG
8 Ed4           GTTCTCCCGC---AACTCTGGCGTATCCGC TCACGGTTCGCGAAG
9 Inv2          GTTCTCCCGC---AACTCTGGCGTATCCGC TCACGGTTCGCGAAG
10Inv1          GTTCTCCCGC---AACTCTGGCGTATCCGC TCACGGTTCGCGAAG

              136   *      *           *                      180
1 Inv4          AGTGACAACAATTCG AACGCAACTTTGAAC GCTGAAGCGTCTCGG
2 Elg2          AGTGACAACAATTCG AACGCAACTTTGAAC GCTGAAGCGTCTCGG
3 Elg1          AGTGACAACAATTCG AACGCAACTTTGAAC GCTGAAGCGTCTCGG
4 Ed2           AGTGGCAACAATTCG AACGCAACTTTGAAC GCTGAAGCGTCTCGG
5 Ed1           AGTGACAACAATTCG AACGCAACTTTGAAC GCTGAAGCGTCTCGG
6 Ed3           AGTGACAACAAATCG AACGCAACTTTGAAC GCTGAAGCGTCTCGG
7 Inv3          AGTGACAACAATTCG AACGCAAATTTGAAC GCTGAAGCGTCTCGG
8 Ed4           AGTGACAACAATTCG AACGCAACTTTGAAC GCTGAAGCGTCTCGG
9 Inv2          AGTGACAACAATTCG AACGCAACTTTGAAC GCTGAAGCGTCTCGG
10Inv1          AGTGACAACAATTCG AACGCAACTTTGAAC GCTGAAGCGTCTCGG
```

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          181                                                  *   225
1 Inv4      TACCTCATGGGTGTG CCTATTTATTCCCCG GCCAACAACATGGCA
2 Elg2      TACCTCATGGGTGTG CCTATTTATTCCCCG GCCAACAACAAGGCA
3 Elg1      TACCTCATGGGTGTG CCTATTTATTCCCCG GCCAACAACATGGCA
4 Ed2       TACCTCATGGGTGTG CCTATTTATTCCCCG GCCAACAACATGGCA
5 Ed1       TACCTCATGGGTGTG CCTATTTATTCCCCG GCCAACAACATGGCA
6 Ed3       TACCTCATGGGTGTG CCTATTTATTCCCCG GCCAACAACATGGCA
7 Inv3      TACCTCATGGGTGTG CCTATTTATTCCCCG GCCAACAACATGGCA
8 Ed4       TACCTCATGGGTGTG CCTATTTATTCCCCG GCCAACAACATGGCA
9 Inv2      TACCTCATGGGTGTG CCTATTTATTCCCCG GCCAACAACATGGCA
10Inv1      TACCTCATGGGTGTG CCTATTTATTCCCCG GCCAACAACATGGCA

          226                                                  270
1 Inv4      AGTGGGAGGTCTCTT TTGAGGCTAGGCTTG AAGAGGGGGTCTCAC
2 Elg2      AGTGGGAGGTCTCTT TTGAGGCTAGGCTTG AAGAGGGGGTCTCAC
3 Elg1      AGTGGGAGGTCTCTT TTGAGGCTAGGCTTG AAGAGGGGGTCTCAC
4 Ed2       AGTGGGAGGTCTCTT TTGAGGCTAGGCTTG AAGAGGGGGTCTCAC
5 Ed1       AGTGGGAGGTCTCTT TTGAGGCTAGGCTTG AAGAGGGGGTCTCAC
6 Ed3       AGTGGGAGGTCTCTT TTGAGGCTAGGCTTG AAGAGGGGGTCTCAC
7 Inv3      AGTGGGAGGTCTCTT TTGAGGCTAGGCTTG AAGAGGGGGTCTCAC
8 Ed4       AGTGGGAGGTCTCTT TTGAGGCTAGGCTTG AAGAGGGGGTCTCAC
9 Inv2      AGTGGGAGGTCTCTT TTGAGGCTAGGCTTG AAGAGGGGGTCTCAC
10Inv1      AGTGGGAGGTCTCTT TTGAGGCTAGGCTTG AAGAGGGGGTCTCAC

          271                                                  315
1 Inv4      CGACCTCGCCGCCCC GTTGGTGCCTTGACC CGGTGGGGGTCTCTG
2 Elg2      CGACCTCGCCGCCCC GTTGGTGCCTTGACC CGGTGGGGGTCTCTG
3 Elg1      CGACCTCGCCGCCCC GTTGGTGCCTTGACC CGGTGGGGGTCTCTG
4 Ed2       CGACCTCGCCGCCCC GTTGGTGCCTTGACC CGGTGGGGGTCTCTG
5 Ed1       CGACCTCGCCGCCCC GTTGGTGCCTTGACC CGGTGGGGGTCTCTG
6 Ed3       CGACCTCGCCGCCCC GTTGGTGCCTTGACC CGGTGGGGGTCTCTG
7 Inv3      CGACCTCGCCGCCCC GTTGGTGCCTTGACC CGGTGGGGGTCTCTG
8 Ed4       CGACCTCGCCGCCCC GTTGGTGCCTTGACC CGGTGGGGGTCTCTG
9 Inv2      CGACCTCGCCGCCCC GTTGGTGCCTTGACC CGGTGGGGGTCTCTG
10Inv1      CGACCTCGCCGCCCC GTTGGTGCCTTGACC CGGTGGGGGTCTCTG

          316                                  *               360
1 Inv4      GAGTATTAGTCCCGG GGACTATCGGACTTG GAAATCTTGTCCGTC
2 Elg2      GAGTATTAGTCCCGG GGACTATCGGACTCG GAAATCTTGTCCGTC
3 Elg1      GAGTATTAGTCCCGG GGACTATCGGACTTG GAAATCTTGTCCGTC
4 Ed2       GAGTATTAGTCCCGG GGACTATCGGACTTG GAAATCTTGTCCGTC
5 Ed1       GAGTATTAGTCCCGG GGACTATCGGACTTG GAAATCTTGTCCGTC
6 Ed3       GAGTATTAGTCCCGG GGACTATCGGACTTG GAAATCTTGTCCGTC
7 Inv3      GAGTATTAGTCCCGG GGACTATCGGACTTG GAAATCTTGTCCGTC
8 Ed4       GAGTATTAGTCCCGG GGACTATCGGACTTG GAAATCTTGTCCGTC
9 Inv2      GAGTATTAGTCCCGG GGACTATCGGACTTG GAAATCTTGTCCGTC
10Inv1      GAGTATTAGTCCCGG GGACTATCGGACTTG GAAATCTTGTCCGTC
```

```
          361                       *                          405
 1 Inv4     TCTGGAGTGCCCTTT CCGTGAATAAGACTC ATCCCCGGACCTTCC
 2 Elg2     TCTGGAGTGCCCTTT CCGTGAACAAGACTC ATCCCCGGACCTTCC
 3 Elg1     TCTGGAGTGCCCTTT CCGTGAACAAGACTC ATCCCCGGACCTTCC
 4 Ed2      TCTGGAGTGCCCTTT CCGTGAACAAGACTC ATCCCCGGACCTTCC
 5 Ed1      TCTGGAGTGCCCTTT CCGTGAACAAGACTC ATCCCCGGACCTTCC
 6 Ed3      TCTGGAGTGCCCTTT CCGTGAACAAGACTC ATCCCCGGACCTTCC
 7 Inv3     TCTGGAGTGCCCTTT CCGTGAACAAGACTC ATCCCCGGACCTTCC
 8 Ed4      TCTGGAGTGCCCTTT CCGTGAACAAGACTC ATCCCCGGACCTTCC
 9 Inv2     TCTGGAGTGCCCTTT CCGTGAACAAGACTC ATCCCCGGACCTTCC
10Inv1      TCTGGAGTGCCCTTT CCGTGAACAAGACTC ATCCCCGGACCTTCC

          406                 *  *   *                    450
 1 Inv4     TAACGGAAGAAATCC TAAATTTTATAAAAT CTCGAACGCTAATAA
 2 Elg2     TAACGGAAGAAATCC TAAATTTTATAAAAT CTCGAACGCTAATAA
 3 Elg1     TAACGGAAGAAATCC TAAATTTTATAAAAT CTCGAACGCTAATAA
 4 Ed2      TAACGGAAGAAATCC TAAATTTTATAAAAT CTCGAACGCTAATAA
 5 Ed1      TAACGGAAGAAATCC TAAATTTTATAAAAT CTCGAACGCTAATAA
 6 Ed3      TAACGGAAGAAATCC TAAATTTTATAAAAT CTCGAACGCTAATAA
 7 Inv3     TAACGGAAGAAATCC TGAATTTTATAAAAT CTCGAACGCTAATAA
 8 Ed4      TAACGGAAGAAATCC TAAATTTTATAAAAT CTCGAACGCTAATAA
 9 Inv2     TAACGGAAGAAATCC TAAATTTTATAAAAT CTCGAACGCTAATAA
10Inv1      TAACGGAAGAAATCC TAAAATTT-TAAAAT CTCGAACGCTAATAA

                                                  ITS1 < > 5.8S gene
          451                       *                          495
 1 Inv4     GGCTTCCGTGAGGTT GCCGAAACTTGACAA CTCTGAG CAGTGGAT
 2 Elg2     GGCTTCCGTGAGGTT GCCGAAACTTGACAA CTCTGAG CAGTGGAT
 3 Elg1     GGCTTCCGTGAGGTT GCCGAGACTTGACAA CTCTGAG CAGTGGAT
 4 Ed2      GGCTTCCGTGAGGTT GCCGAAACTTGACAA CTCTGAG CAGTGGAT
 5 Ed1      GGCTTCCGTGAGGTT GCCGAAACTTGACAA CTCTGAG CAGTGGAT
 6 Ed3      GGCTTCCGTGAGGTT GCCGAAACTTGACAA CTCTGAG CAGTGGAT
 7 Inv3     GGCTTCCGTGAGGTT GCCGAAACTTGACAA CTCTGAG CAGTGGAT
 8 Ed4      GGCTTCCGTGAGGTT GCCGAAACTTGACAA CTCTGAG CAGTGGAT
 9 Inv2     GGCTTCCGTGAGGTT GCCGAAACTTGACAA CTCTGAG CAGTGGAT
10Inv1      GGCTTCCGTGAGGTT GCCGAAACTTGACAA CTCTGAG CAGTGGAT

                          > 5.8S primer
          496                             *     525
 1 Inv4     CACTCGGCTCAC GGA TCGATGAAGACCGCA
 2 Elg2     CACTCGGCTCAC GGA TCGATGAAGACCGCA
 3 Elg1     CACTCGGCTCAC GGA TCGATGAAGACCGCA
 4 Ed2      CACTCGGCTCAC GGA TCGATGAAGACCGCA
 5 Ed1      CACTCGGCTCAC GGA TCGATGAAGACCGCA
 6 Ed3      CACTCGGCTCAC GGA TCGATGAAGACCGCA
 7 Inv3     CACTCGGCTCAC GGA TCGATGAAAACCGCA
 8 Ed4      CACTCGGCTCAC GGA TCGATGAAGACCGCA
 9 Inv2     CACTCGGCTCAC GGA TCGATGAAGACCGCA
10Inv1      CACTCGGCTCAC GGA TCGATGAAGACCGCA
```

5.3.4.5. *Lepthyphantes tenuis* ITS1

The *L.tenuis* ITS1 region has a consensus length of 394 bases, with all clones identical in size. There are five single base pair substitutions - four transitions and one transversion, giving a total sequence variation in the region of 1.3 % (or 5/394 bases) (Figure 5.7). The average number of substitutions per site in each *L.tenuis* ITS1 sequence was calculated as 0.0026 via Kimura's two-parameter method.

Figure 5.7. Multiple alignment of the cloned ITS1 sequences from *Lepthyphantes tenuis*. Eng = England, Sco = Scotland, NZ = New Zealand. Bold represents a substitution, - represents a deletion or gap. An asterisk highlights a site of mutation. The positions of the ITS1 region, 18S and 5.8S gene are indicated.

```
             1                                                   45
                                                  18S gene <  >ITS1
1 Eng2       -AGGTTTGCCGTAGG TGAACCTGCGGCAGG ATCATTATCGA AATT
2 NZ3        --------------- --------CGGCAGG ATCATTATCGA AATT
3 NZ2        -AGGTTTGCCGTAGG TGAACCTGCGGCAGG ATCATTATCGA AATT
4 NZ1        -AGGTTTGCCGTAGG TGAACCTGCGGCAGG ATCATTATCGA AATT
5 NZ4        -AGGTTTGCCGTAGG TGAACCTTCGGCAGG ATCATTATCGA AATT
6 Scot3      -AGGTTTGCCGTAGG TGAACCTGCGGCAGG ATCATTATCGA AATT
7 Eng1       -AGGTTTGCCGTAGG TGAACCTGCGGCAGG ATCATTATCGA AATT
8 Sco1       -AGGTTTGCCGTAGG TGAACCTGCGGCAGG ATCATTATCGA AATT
9 Scot2      -AGGTTTGCCGTAGG TGAACCTGCGGCAGG ATCATTATCGA AATT
10Eng3       -AGGTTTGCCGTAGG TGAACCTGCGGCAGG ATCATTATCGA AATT

            46                                                  90
1 Eng2       TTCCGTGACGGAATA TTCGGTAGAATTCCA CGGTTCTCGATGAGT
2 NZ3        TTCCGTGACGGAATA TTCGGTAGAATTCCA CGGTTCTCGATGAGT
3 NZ2        TTCCGTGACGGAATA TTCGGTAGAATTCCA CGGTTCTCGATGAGT
4 NZ1        TTCCGTGACGGAATA TTCGGTAGAATTCCA CGGTTCTCGATGAGT
5 NZ4        TTCCGTGACGGAATA TTCGGTAGAATTCCA CGGTTCTCGATGAGT
6 Sco3       TTCCGTGACGGAATA TTCGGTAGAATTCCA CGGTTCTCGATGAGT
7 Eng1       TTCCGTGACGGAATA TTCGGTAGAATTCCA CGGTTCTCGATGAGT
8 Sco1       TTCCGTGACGGAATA TTCGGTAGAATTCCA CGGTTCTCGATGAGT
9 Sco2       TTCCGTGACGGAATA TTCGGTAGAATTCCA CGGTTCTCGATGAGT
10Eng3       TTCCGTGACGGAATA TTCGGTAGAATTCCA CGGTTCTCGATGAGT
```

```
            91                                                  * 135
1 Eng2        TCCGTGGCGGAGCCA TGTGTCCCACCGTGA CGGAGGGATCGGTTC
2 NZ3         TCCGTGGCGGAGCCA TGTGTCCCACCGTGA CGGAGGGATCGGTTC
3 NZ2         TCCGTGGCGGAGCCA TGTGTCCCACCGTGA CGGAGGGATCGGTTC
4 NZ1         TCCGTGGCGGAGCCA TGTGTCCCACCGTGA CGGAGGGATCGGCTC
5 NZ4         TCCGTGGCGGAGCCA TGTGTCCCACCGTGA CGGAGGGATCGGTTC
6 Sco3        TCCGTGGCGGAGCCA TGTGTCCCACCGTGA CGGAGGGATCGGTTC
7 Eng1        TCCGTGGCGGAGCCA TGTGTCCCACCGTGA CGGAGGGATCGGTTC
8 Sco1        TCCGTGGCGGAGCCA TGTGTCCCACCGTGA CGGAGGGATCGGTTC
9 Sco2        TCCGTGGCGGAGCCA TGTGTCCCACCGTGA CGGAGGGATCGGTTC
10Eng3        TCCGTGGCGGAGCCA TGTGTCCCACCGTGA CGGAGGGATCGGTTC

           136                                *                180
1 Eng2        CATTCGAACGCGCTA CGGATGCCTTTACCT TAGGAAGCTTTGCTT
2 NZ3         CATTCGAACGCGCTA CGGATGCCTTTACCT TAGGAAGCTTTGCTT
3 NZ2         CATTCGAACGCGCTA CGGATGCCTTTACCT TAGGAAGCTTTGCTT
4 NZ1         CATTCGAACGCGCTA CGGATGCCTTTACCT TAGGAAGCTTTGCTT
5 NZ4         CATTCGAACGCGCTA CGGATGCCTTTACCT TAGGAAGCTTTGCTT
6 Sco3        CATTCGAACGCGCTA CGGATGCCTTTACCT TAGGAAGCTTTGCTT
7 Eng1        CATTCGAACGCGCTA CGGATGCCTTTACCC TAGGAAGCTTTGCTT
8 Sco1        CATTCGAACGCGCTA CGGATGCCTTTACCT TAGGAAGCTTTGCTT
9 Sco2        CATTCGAACGCGCTA CGGATGCCTTTACCT TAGGAAGCTTTGCTT
10Eng3        CATTCGAACGCGCTA CGGATGCCTTTACCT TAGGAAGCTTTGCTT

           181 *                                               225
1 Eng2        TCAATCGGCCGTATC GGGAGCGATAAGTGA GCTTCCCGTCGGCTG
2 NZ3         TCAATCGGCCGTATC GGGAGCGATAAGTGA GCTTCCCGTCGGCTG
3 NZ2         TCGATCGGCCGTATC GGGAGCGATAAGTGA GCTTCCCGTCGGCTG
4 NZ1         TCAATCGGCCGTATC GGGAGCGATAAGTGA GCTTCCCGTCGGCTG
5 NZ4         TCAATCGGCCGTATC GGGAGCGATAAGTGA GCTTCCCGTCGGCTG
6 Sco3        TCAATCGGCCGTATC GGGAGCGATAAGTGA GCTTCCCGTCGGCTG
7 Eng1        TCAATCGGCCGTATC GGGAGCGATAAGTGA GCTTCCCGTCGGCTG
8 Sco1        TCAATCGGCCGTATC GGGAGCGATAAGTGA GCTTCCCGTCGGCTG
9 Sco2        TCAATCGGCCGTATC GGGAGCGATAAGTGA GCTTCCCGTCGGCTG
10Eng3        TCAATCGGCCGTATC GGGAGCGATAAGTGA GCTTCCCGTCGGCTG

           226                                                 *270
1 Eng2        TTTGGTGTGCCTAAT CTTTATTCCCCGGCC GCGACTCGGCAAGAG
2 NZ3         TTTGGTGTGCCTAAT CTTTATTCCCCGGCC GCGACTCGGCAAGAG
3 NZ2         TTTGGTGTGCCTAAT CTTTATTCCCCGGCC GCGACTCGGCAAGAG
4 NZ1         TTTGGTGTGCCTAAT CTTTATTCCCCGGCC GCGACTCGGCAAGAG
5 NZ4         TTTGGTGTGCCTAAT CTTTATTCCCCGGCC GCGACTCGGCAAGAG
6 Sco3        TTTGGTGTGCCTAAT CTTTATTCCCCGGCC GCGACTCGGCAAGAG
7 Eng1        TTTGGTGTGCCTAAT CTTTATTCCCCGGCC GCGACTCGGCAAGGG
8 Sco1        TTTGGTGTGCCTAAT CTTTATTCCCCGGCC GCGACTCGGCAAGAG
9 Sco2        TTTGGTGTGCCTAAT CTTTATTCCCCGGCC GCGACTCGGCAAGAG
10Eng3        TTTGGTGTGCCTAAT CTTTATTCCCCGGCC GCGACTCGGCAAGAG
```

```
          271                                                   315
1 Eng2      GGGGGTCCCTTCACT GGGGCTAGGCTCAAA GAGATGCGCGAAGCC
2 NZ3       GGGGGTCCCTTCACT GGGGCTAGGCTCAAA GAGATGCGCGAAGCC
3 NZ2       GGGGGTCCCTTCACT GGGGCTAGGCTCAAA GAGATGCGCGAAGCC
4 NZ1       GGGGGTCCCTTCACT GGGGCTAGGCTCAAA GAGATGCGCGAAGCC
5 NZ4       GGGGGTCCCTTCACT GGGGCTAGGCTCAAA GAGATGCGCGAAGCC
6 Sco3      GGGGGTCCCTTCACT GGGGCTAGGCTCAAA GAGATGCGCGAAGCC
7 Eng1      GGGGGTCCCTTCACT GGGGCTAGGCTCAAA GAGATGCGCGAAGCC
8 Sco1      GGGGGTCCCTTCACT GGGGCTAGGCTCAAA GAGATGCGCGAAGCC
9 Sco2      GGGGGTCCCTTCACT GGGGCTAGGCTCAAA GAGATGCGCGAAGCC
10Eng3      GGGGGTCCCTTCACT GGGGCTAGGCTCAAA GAGATGCGCGAAGCC

          316                                                   360
1 Eng2      GCACCGCACCTTTGG CGTCCCTTCTCCTCG GAGAAGTGTCTTAAT
2 NZ3       GCACCGCACCTTTGG CGTCCCTTCTCCTCG GAGAAGTGTCTTAAT
3 NZ2       GCACCGCACCTTTGG CGTCCCTTCTCCTCG GAGAAGTGTCTTAAT
4 NZ1       GCACCGCACCTTTGG CGTCCCTTCTCCTCG GAGAAGTGTCTTAAT
5 NZ4       GCACCGCACCTTTGG CGTCCCTTCTCCTCG GAGAAGTGTCTTAAT
6 Sco3      GCACCGCACCTTTGG CGTCCCTTCTCCTCG GAGAAGTGTCTTAAT
7 Eng1      GCACCGCACCTTTGG CGTCCCTTCTCCTCG GAGAAGTGTCTTAAT
8 Sco1      GCACCGCACCTTTGG CGTCCCTTCTCCTCG GAGAAGTGTCTTAAT
9 Sco2      GCACCGCACCTTTGG CGTCCCTTCTCCTCG GAGAAGTGTCTTAAT
10Eng3      GCACCGCACCTTTGG CGTCCCTTCTCCTCG GAGAAGTGTCTTAAT

          361                                                   405
1 Eng2      ACCAACTATCTTTGA ACGCTATACGACGAA CGCCCTCGTGAGGGG
2 NZ3       ACCAACTATCTTTGA ACGCTATACGACGAA CGCCCTCGTGAGGGG
3 NZ2       ACCAACTATCTTTGA ACGCTATACGACGAA CGCCCTCGTGAGGGG
4 NZ1       ACCAACTATCTTTGA ACGCTATACGACGAA CGCCCTCGTGAGGGG
5 NZ4       ACCAACTATCTTTGA ACGCTATACGACGAA CGCCCTCGTGAGGGG
6 Sco3      ACCAACTATCTTTGA ACGCTATACGACGAA CGCCCTCGTGAGGGG
7 Eng1      ACCAACTATCTTTGA ACGCTATACGACGAA CGCCCTCGTGAGGGG
8 Sco1      ACCAACTATCTTTGA ACGCTATACGACGAA CGCCCTCGTGAGGGG
9 Sco2      ACCAACTATCTTTGA ACGCTATACGACGAA CGCCCTCGTGAGGGG
10Eng3      ACCAACTATCTTTGA ACGCTATACGACGAA CGCCCTCGTGAGGGG

          406        *                ITS1< >5.8S    *    450
1 Eng2      GCGCGAGTCACATCG AATGACAACTCTGAA CAGTGGATCACTCGG
2 NZ3       GCGCGAGTCACATCG AATGACAACTCTGAA CAGTGGATCACTCGG
3 NZ2       GCGCGAGTCACATCG AATGACAACTCTGAA CAGTGGATCACTCGG
4 NZ1       GCGCGAGTCACATCG AATGACAACTCTGAA CAGTGGATCACTCGG
5 NZ4       GCGCGAGTCACATCG AATGACAACTCTGAA CAGTGGATCACTCGG
6 Sco3      GCGCGAGTCACATCG AATGACAACTCTGAA CAGTGGATCACTCGG
7 Eng1      GCGCGAGTCCCATCG AATGACAACTCTGAA CAGTGGATCTCTCGG
8 Sco1      GCGCGAGTCACATCG AATGACAACTCTGAA CAGTGGATCACTCGG
9 Sco2      GCGCGAGTCACATCG AATGACAACTCTGAA CAGTGGATCACTCGG
10Eng3      GCGCGAGTCACATCG AATGACAACTCTGAA CAGTGGATCACTCGG
```

```
          451     > 5.8S primer       475
1 Eng2      CTCAC GGATCGATGA AGACCGCAGC
2 NZ3       CTCAC GGATCGATGA AGACCGCAGC
3 NZ2       CTCAC GGATCGATGA AGACCGCAGC
4 NZ1       CTCAC GGATCGATGA AGACCGCAGC
5 NZ4       CTCAC GGATCGATGA AGACCGCAGC
6 Sco3      CTCAC GGATCGATGA AGACCGCAGC
7 Eng1      CTCAC GGATCGATGA ----------
8 Sco1      CTCAC GGATCGATGA AGACCGCAGC
9 Sco2      CTCAC GGATCGATGA AGACCGCAGC
10Eng3      CTCAC GGATCGATGA AGACCGCAGC
```

5.3.4.6. *Bathyphantes gracilis* ITS1

The three ITS1 cloned sequences (339-341 bases) from the single *B.gracilis* contained seven polymorphisms, representing a total variation of 2.1 % (or 7/341 bases) (Figure 5.8). The length differences were caused by one single base deletion and one double base deletion event, both occurring in a single clone. The Kimura two parameter (1980) ratio of substitutions per site in the *B.gracilis* ITS1 sequences was calculated as 0.0097. In addition to the two deletions, there were three transition and two transversion substitution events. Across all three species the number of transitions outweighed the number of transversions (a total ratio of 13:7), which is the typical pattern found in nucleotide substitutions (Li 1996).

Figure 5.8. Multiple alignment of the cloned ITS1 sequences from *Bathyphantes gracilis*, Edinburgh. Bold represents a substitution, - represents a deletion or gap. An asterisk highlights a site of mutation. The positions of the ITS1 region, 18S and 5.8S gene are indicated.

```
                      1               18S   < > ITS1 30
1 bg1                 CCTGCGGCTGGATCA TTATCGA AACCGGGA
2 bg7                 CCTGCGGCTGGATCA TTATCGA AACCGGGA
3 bg3                 CCTGCGGCTGGATCA TTATCGA AACCGGGA

     31                                                75
1 bg1 TGAAAATTCCAGTAT CGGTGAAACACCTCT CTTCCCCGTCTTCAT
2 bg7 TGAAAATTCCAGTAT CGGTGAAACACCTCT CTTCCCCGTCTTCAT
3 bg3 TGAAAATTCCAGTAT CGGTGAAACACCTCT CTTCCCCGTCTTCAT
```

```
     76   *                                                120
1 bg1 CGGCGGGGTCGAGGG AGAGGCGAGACTGGC TTCACCGCGACGGTG
2 bg7 CGGCGGGGTCGAGGG AGAGGCGAGACTGGC TTCACCGCGACGGTG
3 bg3 CGGCAGGGTCGAGGG AGAGGCGAGACTGGC TTCACCGCGACGGTG

    121                                                165
1 bg1 GAGCGAAGTCTTGCC GTTCGAACGCAATAC GACCCGTCACATGGT
2 bg7 GAGCGAAGTCTTGCC GTTCGAACGCAATAC GACCCGTCACATGGT
3 bg3 GAGCGAAGTCTTGCC GTTCGAACGCAATAC GACCCGTCACATGGT

    166                          *   * *             210
1 bg1 GTGCCTAACTTGTAT TCCCGGCCTTTGACT -TAGGCAGGAGGGAG
2 bg7 GTGCCTAACTTGTAT TCCCGGCCTTTGACC ATAGGCAGGAGGGAG
3 bg3 GTGCCTAACTTGTAT TCCCGGCCTTCGACC ATAGGCAGGAGGGAG

    211                                            *   255
1 bg1 GTCCCGATTCGTCGG GGCTAGGCTTTAAGA GATGCGTCGCAGCAC
2 bg7 GTCCCGATTCGTCGG GGCTAGGCTTTAAGA GATGCGTCGCTGCAC
3 bg3 GTCCCGATTCGTCGG GGCTAGGCTTTAAGA GATGCGTCGCTGCAC

    256                               * *             300
1 bg1 GCACCGCACCGACGG CGTCCCTTCCCTTTA --GGGGAGTGTTTCT
2 bg7 GCACCGCACCGACGG CGTCCCTTCCCTTTT TTGGGGAGTGTTTCT
3 bg3 GCACCGCACCGACGG CGTCCCTTCCCTTTT TTGGGGAGTGTTTCT

    301                                                345
1 bg1 GAAACAAACAAATTT GAACGCACTACGGCC GGGAGGCCTTAATTC
2 bg7 GAAACAAACAAATTT GAACGCACTACGGCC GGGAGGCCTTAATTC
3 bg3 GAAACAAACAAATTT GAACGCACTACGGCC GGGAGGCCTTAATTC

    346                                                 390
                  ITS1< > 5.8S                 > 5.8S primer
1 bg1 TAAATTGACAACTCTGAA CAGTGGATCACT CGGCTCAC GGATCGA
2 bg7 TAAATTGACAACTCTGAA CAGTGGATCACT CGGCTCAC GGATCGA
3 bg3 TAAATTGACAACTCTGAA CAGTGGATCACT CGGCTCAC GGATCGA

    391           403
1 bg1 TGAAGACCGCAGC
2 bg7 TGAAGACCGCAGC
3 bg3 TGAAGACCGCAGC
```

Interestingly, in contrast to the *E.ovata* sequences, none of the substitutions in either *L.tenuis* or *B.gracilis* occurred following an adenine base. Given that all species were amplified using the same *Taq* polymerase, this reduces the possibility of these *E.ovata* polymorphisms having arisen from *Taq* irregularities in terms of incorporation of bases following an adenine, and are more likely to be genuine polymorphisms.

5.3.4.7. Comparison of spider ITS1 regions

A NCBI database search with the spider ITS1 regions, using the default search parameters, unsurprisingly found no matches with other Arachnid ITS regions (there are apparently no spider ITS accessions in the database). Indeed, the three spider species examined in this study cover a relatively broad range of species relatedness, and due to the polymorphic nature of the ITS1 region there is only weak homology apparent between the regions. After CLUSTAL W 1.6 alignment, using the default parameters, and subsequent alignment by eye, the consensus spider ITS1 sequences were compared via the DNADIST sequence analysis programme of PHYLIP. *L.tenuis* and *B.gracilis* shared a much higher degree of evolutionary similarity than either did with *E.ovata* as calculated via the Kimura two parameter distance method (*L.tenuis* and *B.gracilis* had a substitutions per site average of 0.4, whereas *E.ovata* scored 0.55 substitutions per site compared to *B.gracilis*, and 0.73 with *L.tenuis*). These values are in stark contrast to the within species estimates which were all < 0.01 substitutions per site. This relatively low level of inter-specific homology is to be expected given that ITS1 variations between closely related

species are commonly used as diagnostic characters and this study is looking at variation across two families of spider - the Linyphidae and the Theridiidae. Indeed, Vogler and DeSalle (1994) attempted to use sequence data from the ITS1 region from two species of beetle from the same genus, and found the sequences too divergent for comparison.

Interestingly though, there are several blocks of sequence with relatively high homology that are retained between the spider species, particularly between *L.tenuis* and *B.gracilis*, and these may preserve important features of secondary structure when the RNA transcript is folded prior to subsequent processing. This hypothesis was tested by comparing the ITS1 regions of *L.tenuis* and *B.gracilis* (Figure 5.9) and plotting where the regions of similarity occurred on the predicted secondary structure (see Section 5.3.4.8).

Interestingly, a cursory examination of the sequences indicates that the 3' half of the spider ITS1 regions contains a greater proportion of conserved blocks of sequence, and this is in accordance with the inter-specific study of *Drosophila* ITS1 (Schlotterer *et al.* 1994). It would therefore seem likely that the second half of the ITS1 plays more of a functional role than the 5' end in arthropods.

As stated above, the comparison of *L.tenuis* and *B.gracilis* ITS1 regions showed a considerably greater level of homology than either did with *E.ovata*, indicating the closer evolutionary relationship of these two Linyphiid spiders. The high degree of sequence variation between these genera within a family however, in contrast to the high level of similarity of the rDNA arrays within a species, does again emphasise the degree to which concerted evolution has taken place.

Figure 5.9. Comparison of ITS1 regions of *Lepthyphantes tenuis* and *Bathyphantes gracilis*. Bold represents a shared base, - represents a deletion or gap. The asterisks highlight regions of similarity (≥ 6 consecutive bases). The positions of the ITS1 region, 18S and 5.8S gene are indicated.

```
                                        18S          < > ITS1
L.tenuis                    -CCTGCGGCAGGATC ATTATCGA AATTTTC
B.gracilis                  -CCTGCGGCTGGATC ATTATCGA AA----C

L.tenuis    CGTGACGGAATATTC GGTAGAATTCCACGG TTCTCGATGAGTTCC
B.gracilis  CGGGATGAAAATTCC AGTA----------- ---TCGGTGAAACAC

                                          (1)*****
L.tenuis    GTGGCGGAGCCATGT GTCCCACCGTGACGG AGGGATCGGTTCCAT
B.gracilis  CTCTCTTCCCCGTCT TCATCGGCAGGGTCG AGGGAGAGG------

L.tenuis    TCGAACGCGCTACGG ATGCCTTTACCTTAG GAAGCTTTGCTTTCA
B.gracilis  -------CGAGAC-- -TGGCTTCACC--GC GACG-GTGGAGCGAA

                                           (2)****
L.tenuis    ATCGGCCGTATCGGG AGCGATAAGTGAGCT TCCCGTCGGCTGTTT
B.gracilis  GTCTTGCCGTTCGAA CGCAATACGA----- -CCCGTCACATG---

            (3)********    (4)*****
L.tenuis    GGTGTGCCTAATCTT TATTCCCCGGCCGCG AC--TCGGCAAGAGG
B.gracilis  -GTGTGCCTAACTTG TATTCCCGGCCTTCG ACCATAGGCAGGAGG

             (5)****        (6)********* (7)*********
L.tenuis    GGGGTCCC--TTCAC TGGGGCTAGGCTCAA AGAGATGCG-CGAAG
B.gracilis  GAGGTCCCGATTCGT CGGGGCTAGGCTTTA AGAGATGCGTCGCTG

             (8)*********   (9)***********
L.tenuis    C-CGCACCGCACCTT TGGCGTCCCTTCTCC TCG--GAGAAGTGTC
B.gracilis  CACGCACCGCACCGA CGGCGTCCCTTCCCT TTTTTGGGGAGTGTT

                          (10)******
L.tenuis    TTAATACCAACTATC TTTGAACGCTATACG ACGAACGCCCTCGTG
B.gracilis  TCTGAAACAAACAAA TTTGAACGCACTACG GC---------CGGG

                                    (11)******>5.8S
L.tenuis    AGGGGGCGCGAGTCA CATCGAATGACAACT CTGAA CAGTGGATCA
B.gracilis  AGG--CCTTAATTCT AA---ATTGACAACT CTGAA CAGTGGATCA

L.tenuis    CTCGGCTCAC
B.gracilis  CTCGGCTCAC
```

5.3.4.8. Secondary structure of ITS1 regions

The predicted secondary structure of the spider ITS1 regions was constructed using the "Mfold" programme (Zucker 1989) available on the MacFarlane Burnet Centre Mfold on-line server (http://mfold.mbcmr.unimelb.edu.au/), using the default parameters. As suggested by Yeh *et al.* (1990) the last 52 nucleotides of the 18S gene and the first 50 nucleotides of the 5.8S gene were also included in the folding as these are considered integral parts of the secondary structure. Using this approach, minimal energy folding patterns were obtained, and compared for all three spider species. Notably though, this is a minimum energy conformation, and a different conformation may be obtained if the secondary structure was determined by interactions with proteins. However, despite that reservation, the creation of a hypothetical secondary structure allows further investigation of mutational events and their possible structural consequences.

The Mfold programme generates a number of possible secondary structures. The range of folding energies for the ITS1 of each species was as follows ; *L.tenuis*, -160.7 to -169.0 kcal/mol, *B.gracilis*, -157.0 to -164.9 kcal/mol, and *E.ovata*, -164.0 to -171.4 kcal/mol.

The folding patterns were examined and compared between species, and examples chosen which contained the conserved stem-loop structures I, II and III present in other eukaryotic organisms (Yeh *et al.* 1990). These common structures

correspond to the beginning of the ITS1 region (I), the end of the ITS1 region (III), and a loop found consistently near loop I (II) (Figures 5.10-5.12).

The predicted folding of all three species shares a high degree of structural similarity, despite the differing lengths of the ITS1 region. For example, of the 11 regions of prominent homology shared between the ITS1 sequence of *L.tenuis* and *B.gracilis* presented in Figure 5.9 (i.e. ≥ 6 consecutive bases, numbered and highlighted by asterisks above the sequence), ten were found to occur on stems and only one (region 8) occurred at a loop. Indeed, even this region helped to create very similar structures, indicating that these regions may indeed play a role in maintaining secondary structure. Furthermore, the conserved blocks 4-10 help create four distinct stem-loop structures, present in *L.tenuis* and *B.gracilis*, at least two of which can also be found on the *E.ovata* secondary structure (in this case blocks 3, 4 and 6 maintain the stem-loops). The presence of these structural similarities across two spider families is quite compelling circumstantial evidence that the ITS1 region plays an important functional role in processing the genic components of the transcript.

In addition, by mapping the *E.ovata* and *B.gracilis* intra-specific mutations onto their respective proposed structures it was found that in general (8/12 and 5/7 respectively), they were occurring in regions least likely to cause disruption of the overall structure (i.e. in loops or in un-paired regions) which do not contribute to intramolecular base pairing. For example, in the *E.ovata* ITS1 sequence from Elgin the insert at base 101-103 could be incorporated into the un-paired region (base 113 on figure) and the single base deletion is accommodated in a loop (437-441 on

figure). The majority of the other mutation events took place in stem-loops (e.g. base 100 and 150), or unpaired regions (e.g. base 354 and 432). In the case of the *B.gracilis* "hot spots", the two substitutions and base deletion between bases 192-197 can be incorporated in a stem-loop (bases 219-228 on figure) and the 2 base deletion and substitution (bases 285-287) are accommodated in the stem-loop (312-318 in figure). The majority of *L.tenuis* intra-specific substitutions (3/5) events also occur in unpaired positions on the hypothetical secondary structure.

This analysis obviously pre-supposes that it is the secondary structure of the stem, rather than the loop, which is important for the subsequent processing of the folded transcript. This is supported by the work of van der Sande *et al.* (1992) who reached this conclusion from functional analysis of yeast ITS2 sequences.

To summarise, whilst not conclusive, taking both the conserved structure and position of mutations into consideration, these predicted secondary structures appear on balance to represent their respective ITS1 regions, and add weight to the supposition that the increased numbers of intra-specific polymorphisms grouped in apparent "hot spots" may be real areas of low selection pressure rather than PCR artefact.

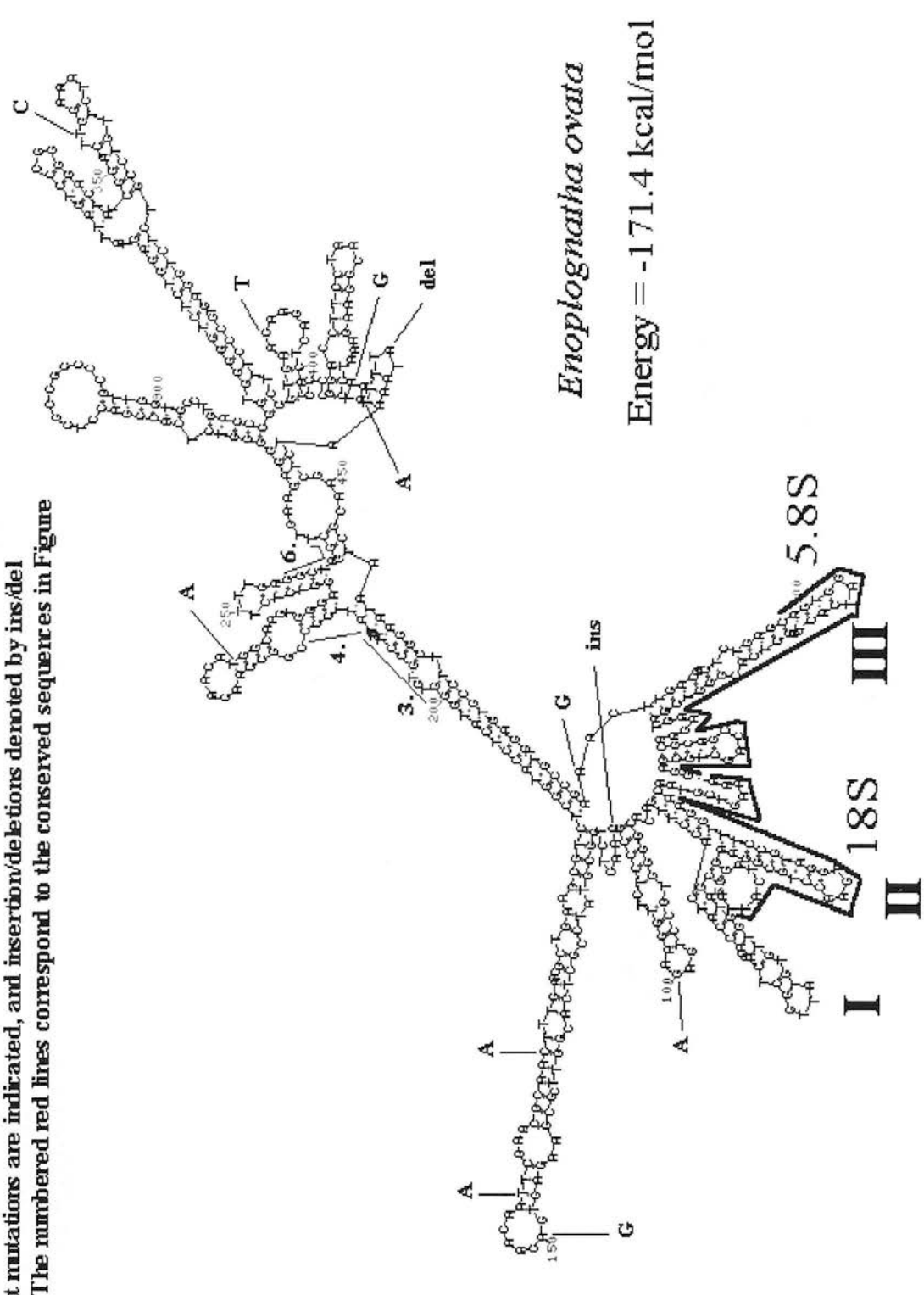


Figure 5.10. Secondary structure of *Enoplognatha ovata* ITS1 region
The 5.8S and 18S genes are indicated. The conserved loops I, II and III are also labelled. Point mutations are indicated, and insertion/deletions denoted by ins/del respectively. The numbered red lines correspond to the conserved sequences in Figure 5.9.

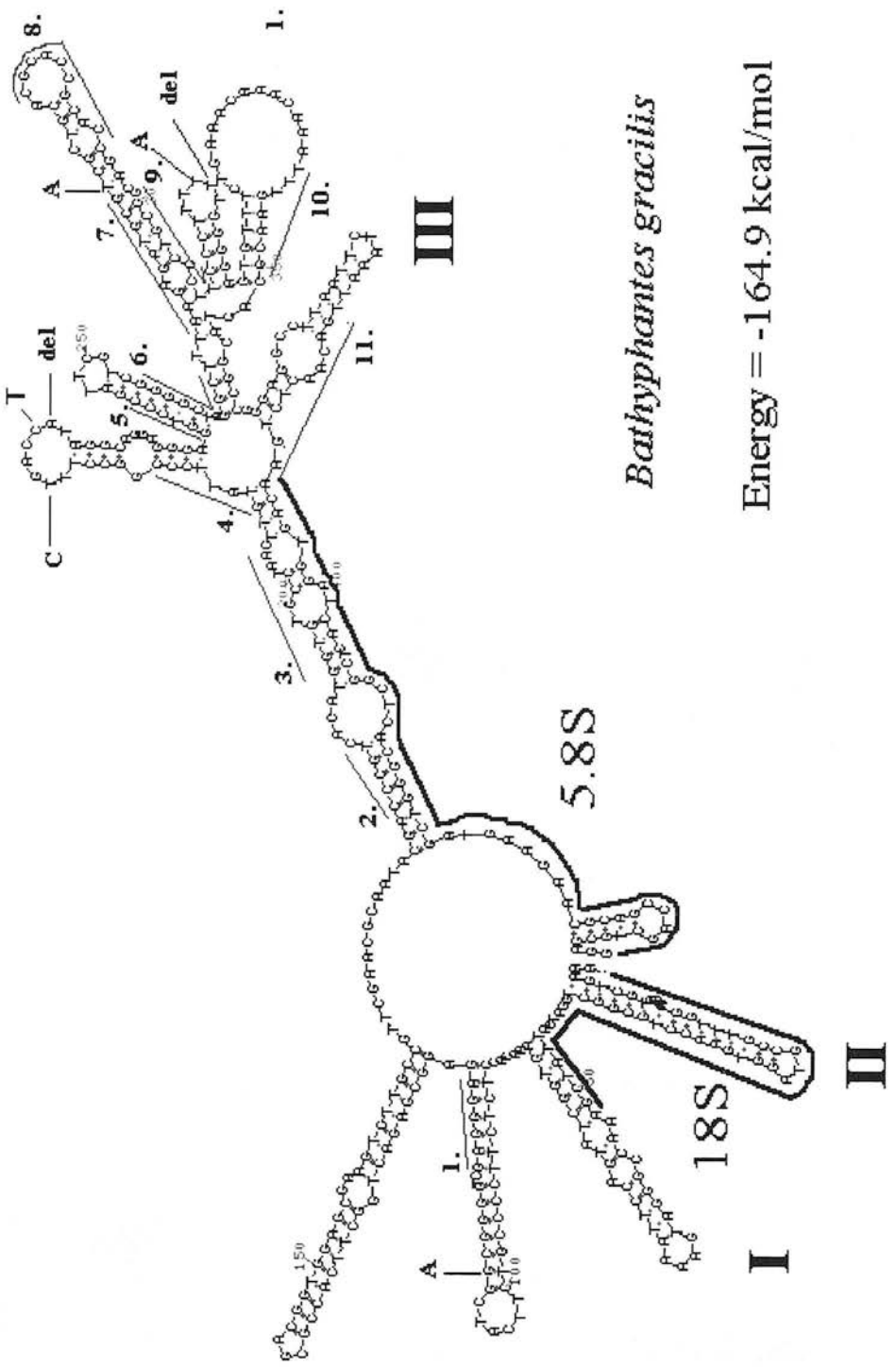
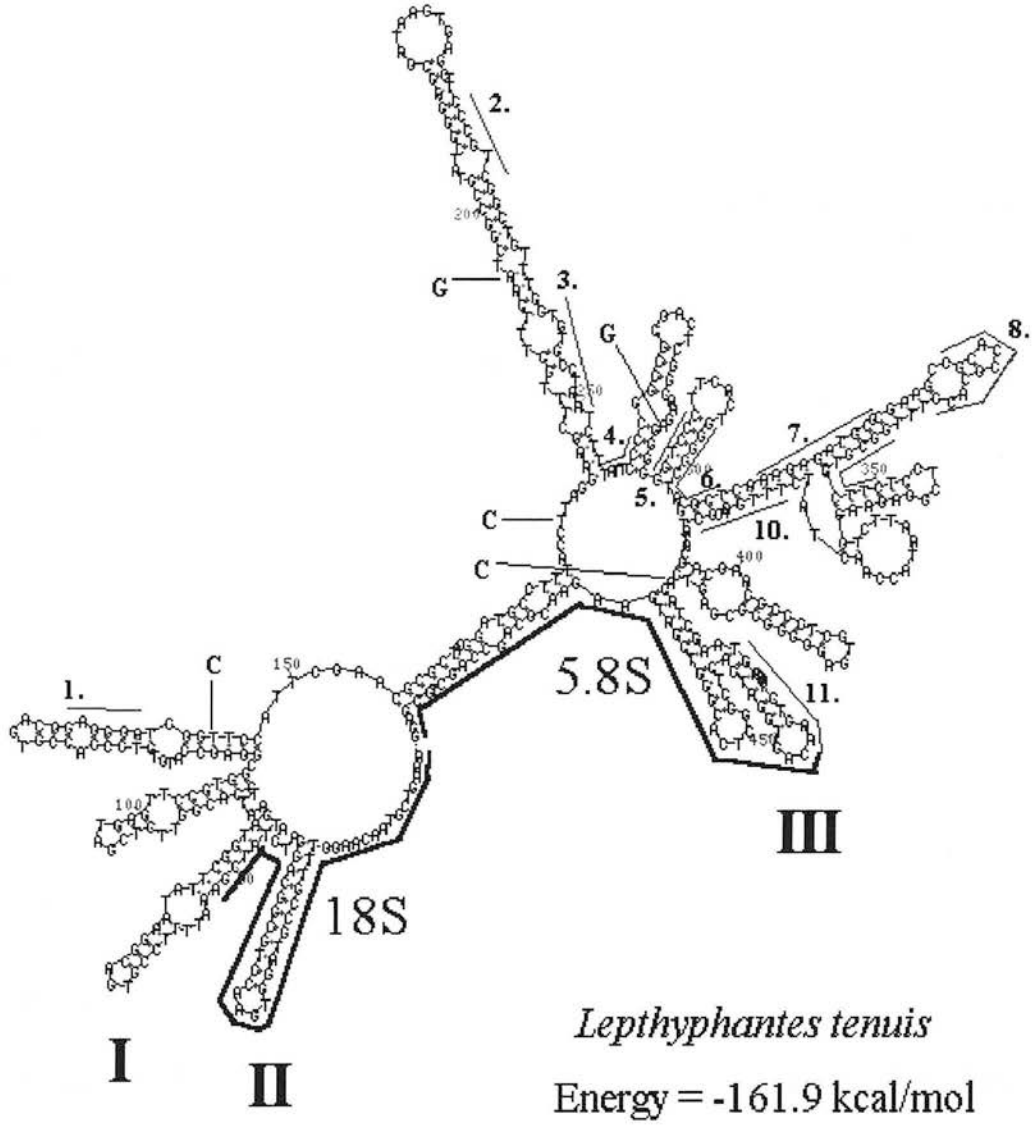


Figure 5.11. Secondary structure of *Bathyphantes gracilis* ITS1 region
The 5.8S and 18S genes are indicated. The conserved loops I, II and III are also labelled. Point mutations are indicated, and insertion/deletions denoted by ins/del respectively. The numbered red lines correspond to the conserved sequences in Figure 5.9.

Figure 5.12. Secondary structure of *Lepthyphantes tenuis* ITS1 region
The 5.8S and 18S genes are indicated. The conserved loops I, II and III are also labelled. Point mutations are indicated, and insertion/deletions denoted by ins/del respectively. The numbered red lines correspond to the conserved sequences in Figure 5.9.



The possibility of *Taq* polymerase nucleotide mis-incorporations, due to the polymerase lacking an editing function (Sambrook *et al.* 1989), must be considered when dealing with sequences derived from PCR generated DNA fragments, since PCR errors are known to create minor differences in sequences with a frequency of transition errors of approximately one per 500 bases (0.025 %) (McLain *et al.* 1995). The presence of a base substitution in the conserved sequence of the 5.8S gene of *L.tenuis* would seem to indicate that a degree of error is occurring, as it is highly unlikely that a conserved gene such as this is genuinely variable within a species. Moreover, SSR sequences have been shown to increase in length when amplified by various DNA polymerases *in vitro* (Schlotterer and Tautz 1991). Indeed, although point mutations rather than repeat duplications were found in the *E.ovata* sequences following the CAA motif, perhaps something about the motif may contribute to slippage and mis-incorporation errors (although whether this is occurring *in vitro* or *in vivo* would be difficult to ascertain without direct cloning avoiding PCR). A second possible source of error arises from the fact that the ITS1 sequences were only read in one, the "forward" 5'-3', direction. It is possible that some of the putative polymorphisms would be resolved by sequencing in the other direction.

Whilst taking both of these possibilities into account, the level of polymorphism which has been found is in excess of that accountable by PCR error. Furthermore, there is a slightly higher level of variation found in the *E.ovata* ITS1 region when compared with *L.tenuis*. Interestingly, following completion of the practical work in this study, Fenton *et al.* (in press) produced a paper which highlighted that the use of "hot start" PCR (a PCR method which involves only

adding the polymerse enzyme to the reaction mix once a high temperature has been reached - thereby avoiding premature annealing and elongation of primers) with an aphid species actually amplified a rare ribosomal haplotype which would otherwise have remained undetected using traditional PCR. This obviously has important implications for this, and indeed all, PCR based studies, and would be investigated seriously in any further spider work.

To assess if there was any correspondence between geographic origin and sequence variation within a species, the PHYLIP software package was used to generate dendrograms to depict graphically relationships between the clones from each population. However, not surprisingly, given the low level of overall variation, there was no degree of geographic separation and the sequences from different locations overlapped extensively (results not shown). As less than 50 variable sites is thought to render a tree relatively inaccurate and we have at best only 12 for *E.ovata* this was not unexpected. In fact, in all three spider species studied, the ITS1 variation was in the form of unique mutations occurring in a single sequence i.e. there is no evidence of different rDNA variants arising within one population (which would have been indicative of concerted evolution having taken place at a local scale) but there is strong evidence of concerted evolution over all populations sampled.

The fact that the sequence variation in both *L.tenuis* and *E.ovata* was limited to a few unique polymorphisms in each clone suggests that all the populations studied either shared a recent ancestor or are currently bound by gene flow. The geographic proximity, within Scotland, of the *E.ovata* samples would at first glance put them into the latter category, but if we consider the RAPD data, presented in Chapter

Four, which shows differentiation over short geographic distances, it is more likely that the similarity is due to a relatively recent ancestor.

The similarity between the *L.tenuis* ITS1 sequences from New Zealand and Britain, despite their distance apart, is perhaps indicative of their introduction from Europe, approximately 250 years ago. It would appear that this length of time has not been sufficient to allow new mutations to occur and become fixed into the New Zealand population. Alternatively, mutations may have occurred but have been quickly selected against and lost from the population. Indeed, negative natural selection may be eliminating deleterious new mutations, or even those which are selectively neutral, in both the British and New Zealand populations, maintaining their high level of similarity. This would occur if the current repeat (shared through a common ancestor) is favoured by positive natural selection. This point emphasises that theoretically the process of molecular drive can both accelerate between species differentiation, whilst maintaining high within species homogeneity, as highlighted by the low variation within but large difference between the linyphiids *L.tenuis* and *B.gracilis*.

The uniformity of the *L.tenuis* sequence data, generated from spiders sampled in an agricultural setting (and hence presumably under selection pressure from agrochemicals) suggests a common factor linking the low levels of variation found in the rDNA of *L.tenuis* and several other agricultural pests (e.g. the Cassava green mite and the Coffee berry borer beetle). However, recent work by Fenton and co-workers (1998 in press) has highlighted rDNA variation in aphids, which indicates that agricultural pressures do not have homogenising effects *per se* on levels of

rDNA variation and so other factors, such as life history strategies, have to be examined.

The limited *B.gracilis* ITS1 data are interesting. *B.gracilis* is also a Linyphiid, but less dispersive than *L.tenuis,* and has an overall level of variation (2.1 %) across the three cloned sequences, higher than that found in *L.tenuis* (1.3 %) but less than that of *E.ovata* (2.7 %). This can tentatively be used to suggest that the lower level of polymorphism was not simply a Linyphiid artefact, and may be suggestive of dispersal affecting homogeneity of rDNA arrays. However, only by sampling populations over a wider species, and geographic, range can this apparent "slowing down" effect of dispersal on concerted evolution be elucidated fully.

Another possibility for the observed low levels of variation across populations may simply be due to the spatial scale studied not being sufficiently large to detect variation. Only very limited gene flow is thought necessary to prevent interlocality differentiation among neutral alleles - exchange of greater than one individual per generation (Slatkin 1987). It would therefore be advantageous to sample specimens from the edges of the species range to ensure there has not been a genetic bottle-neck effect on British spiders which has simply been transferred to New Zealand. As mentioned in Chapter One, it has been hypothesised that *L.tenuis* numbers may have risen sharply in step with the agronomic revolution. It may be that the current British populations have originated from a small genetic base, reflected in the homogeneity of the ITS region. This restricted genetic make-up would then have been transferred to New Zealand. One method for assessing this would be to carry out a parallel study

with mtDNA, which is more effective at tracing historical gene introgressions e.g. Hall and Smith (1991).

This juncture allows the re-introduction of a point raised previously regarding the importance of genetic drift in the fixation of homogenised rDNA arrays. The greater number of point mutations in the *E.ovata* sequences could be indicative of genetic drift beginning to occur in populations of this more sedentary species. As inbreeding increases over generations in small populations the propensity for mutation and genetic drift are thought to increase (Futuyma 1986). The greater number of mutations could represent the effects of genetic drift. In contrast, *L.tenuis* populations are less likely to undergo genetic drift due to their ballooning capabilities and gene flow. Needless to say this is conjecture based on a limited sample size and geographic range, but would nevertheless seem feasible. A further theoretical explanation for the greater number of mutations in *E.ovata* ITS1 clones, also linked with population size, is that gene conversion, the most widely accepted mechanism of concerted evolution, is thought to reach equilibrium faster with a larger population size (Li 1996). *L.tenuis* has a much larger effective population size due to the species ballooning ability, whereas the smaller *E.ovata* populations may be slower to reach homogeneity and as a result, mutations may persist for longer.

One limitation in this study that is acknowledged is the small number of recombinant plasmids that were examined from each individual. Choosing only one individual from a population for analysis is in itself not limiting, since the average individual is thought to contain a high proportion of the rDNA variation present within a population (Learn *et al.* 1987). However, given there are at least several

hundred copies of the rDNA array present in each individual, these results comprise only a small proportion of the total possible variation. From a total of ten *E.ovata*, ten *L.tenuis* and three *B.gracilis* clones, eight, four and three clones respectively were polymorphic for their respective ITS1 sequence. It is therefore unlikely that the total amount of genetic variation within a population, or indeed, even a single individual has been comprehensively described, and would require a substantially larger data set (bearing in mind that typically there are several hundred repeats present in each array). Unfortunately, it was not possible to expand the data set in the present study due to time constraints. However, the information obtained in this study is, I feel, sufficient to give an indication of the main characteristics of the levels of variation of the ITS1 region in the samples of these spider species.

5.3.5. rDNA gene sequence analysis

5.3.5.1. Validation of ribosomal sequences

The partial *E.ovata* sequence generated (approximately 360 bases) for the 28S gene was compared to other invertebrate sequences in the NCBI sequence database, and was found to rank the highest level of homology (85 %) with the tarantula *Eurypelma californica*, which is the only spider 28S sequence in the database. The next closest ranking accessions were also arthropods but with lower levels of homology (< 75 %), validating the origin of the fragment as spider. The 5.8S sequence data were also compared to those present in the database and were found to have the greatest homology (90 %) with the tick *Ixodes*. Again this is

reassuring given that there are no spider 5.8S data in the database and the best match was made with another Arachnid.

The portion of the 18S gene amplified with the primers is very short, some 60 bases, but has 100 % homology with *E.californica* in addition to a number of other arthropods. Given the short length of the sequence and its conserved nature several 100 % matches were expected.

5.3.5.2. Phylogenetic analysis

A detailed phylogeny of the arthropods has previously been constructed by Friedrich and Tautz (1995) using sequences of the 18S and 28S genes from taxa covering the four major extant classes of arthropods - the Myriapoda, Crustacea, Hexapoda and the Chelicerata (the latter included the tarantula - *E.californica*). However, to my knowledge no rDNA-based phylogenetic study has been carried out within the Arachnida. The aim of creating phylogenetic trees using the spider 5.8S and 28S sequence data was to illustrate that these gene sequences could be used more extensively in further studies to address relatedness at this systematic level. The 5.8S dendrograms, produced using both the DNA parsimony and UPGMA tree building algorithms, generated identical tree topology, clearly separating the mollusc, insects, and Arachnids (Figure 5.13).

Two tree building methods were used to add robustness to the results. The methods differ in their approach: UPGMA is based on genetic distance measures, whilst the parsimony approach is based on character states at particular sites. Both methods have their criticisms - UPGMA analysis assumes a constant rate of evolution

of all branches of the tree, and this assumption may not be true. The parsimony approach becomes weaker as the degree of divergence between sequences increases, as several homeoplastic (i.e. parallel, convergent and back (reversal) substitution) events may have taken place. Undertaking both approaches limits the possibility of error as an artefact of the tree building methodology (this conservative multi-methodology approach is endorsed by Avise 1994).

Interestingly, the 5.8S trees both place the tick (*Ixodes*) closer to the spider sequence than to the mites - which are generally considered the tick's closest relative, and the split is well supported as indicated by the bootstrap values. Furthermore, when the analysis is repeated with the longer 28S sequence data (300 bases) a similar picture emerges, with the tick *Boophilus* clustering closer to the spiders than to the mites, which again form a distinct separate group (Figure 5.14). As can be seen by the bootstrap values, the split between the mites and the spiders and tick is also well supported. Within the spiders themselves, the *E.ovata* and *E.californica* grouping is only very weakly supported by the bootstrap values, and this reflects the morphologically-based phylogeny of spiders which would not group these species (Coddington and Levi 1991). However, a note of caution must be added before accepting that the perceived wisdom of arachnid systematics has been turned upside down by this study! A data set of three hundred bases and a few representative arachnid species is clearly not sufficient to say with certainty that the topology of the tree is an accurate reflection of the actual situation. Many more species and a greater quantity of sequence data is required before such a suggestion can be made.

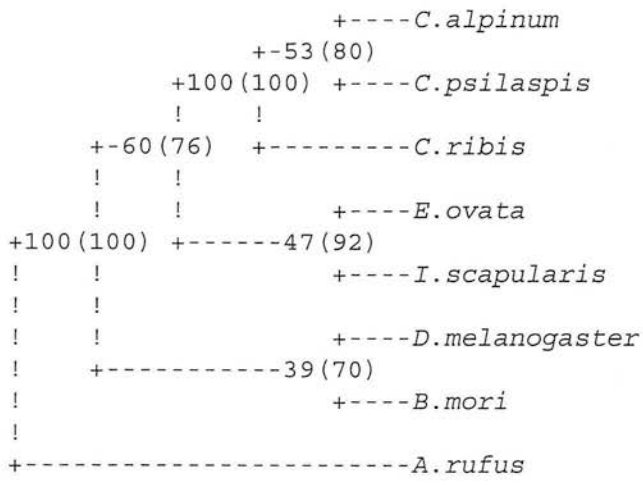


Figure 5.13. Dendrogram produced by parsimony and distance (UPGMA) analysis of 5.8S sequences, reflecting the relationships between the spider *Enoplognatha ovata*, the insects *Drosophila melanogaster* and *Bombyx mori*, the mites *Cecidophyopsis alpinum*, *C.ribis* and *C.psilaspis*, the tick *Ixodes scapularis* and the snail, *Arion rufus*. The percentage of bootstrapped trees supporting the topology are indicated at the branches with parsimony analysis the first value and UPGMA the second value.

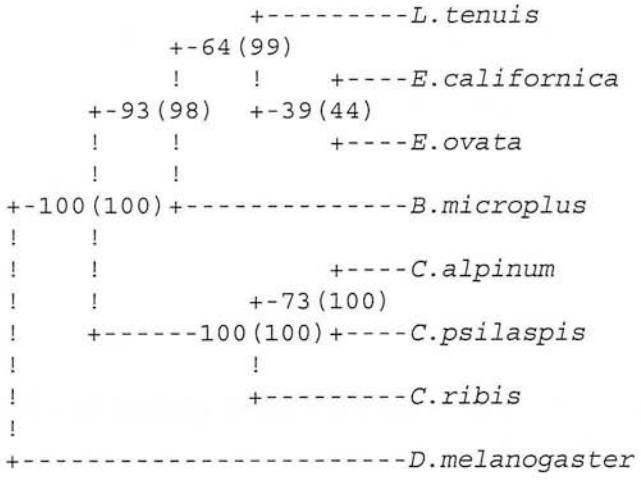


Figure 5.14. Dendrogram produced by parsimony and distance (UPGMA) analysis of 28S sequences, reflecting the relationships between three spider species *Lepthyphantes tenuis*, *Eurypelma californica*, and *Enoplognatha ovata*, a tick *Boophilus microplus*, three mite species *Cecidophyopsis alpinum*, *C.ribis* and *C.psilaspis*, and an insect *Drosophila melanogaster*. The percentage of bootstrapped trees supporting the topology are indicated at the branches with parsimony analysis the first value and UPGMA the second value.

The results do, however, open the arena of arachnid phylogenetics to a more purposeful molecular examination. As Fritz Vollrath, an eminent arachnologist reported in the popular scientific journal, New Scientist, July 1997, "...spider systematics is in a right mess...." Having generated and applied data suitable to begin addressing questions relating to phylogeny within the Arachnida, these preliminary studies re-emphasise the need for an in-depth sequence analysis of the group.

As stated above, the rDNA gene data can be utilised to address questions of relatedness within the Arachnida. To investigate higher resolution relationships within the Araneae, ITS1 sequences could be applied, but would require a more directed effort of sequencing closely related species to test the accuracy of the method. As a preliminary study the aim of which was to examine potential population variation in only a limited number of species this further study was unfortunately impossible, but it would be a relatively simple step to take given the ITS sequence data now obtained. This data could be used to generate spider specific primers, and to use as a comparison. The study has given an indication of the potential of ITS1 in spider systematics by correctly placing the two Linyphiid spiders more closely related to each other than to *E.ovata* (Section 5.3.4.7).

5.4. Evolutionary implications of concerted evolution

Concerted evolution of gene families is very different from the traditional notion of the independent evolution of genes, and it has some very profound evolutionary consequences. The horizontal spreading of a variant repeat to all members of a gene family means that an advantageous mutant repeat can replace all

other repeats and rapidly become fixed in the multigene family. Therefore, through the cellular processes that drive concerted evolution, a small selective advantage can quickly become fixed in a population and become a major advantage (Walsh 1985). Notably, the progressive homogenisation of the rDNA family can give rise to stronger natural selection against further homogenisation - if the specific pattern of folding of the ITS spacers is required for effective processing of the 45S pre-rRNA (as would seem indicated in Section 5.3.4.8). Therefore, drive intensified selection could impede the fixation of a "less fit" mutant at some level of the homogenisation process, whilst not acting to impede the random fixation of a mutation within a subset of "fit" rDNA units possessing a long term advantage. This can explain how rapidly diverging species maintain intra-specific homogeneity of rDNA sequences.

5.5. Summary

The primers and protocols employed in this study were highly successful at amplifying the spider rDNA region, and the size and base composition of the rDNA sequences generated were comparable to those found in other arthropods. RFLP-PCR analysis of the rDNA array did not highlight any obvious polymorphisms between the populations studied. Sequencing was carried out to increase the resolution of the assay. The spider 5.8S and 28S gene sequences were compared to those in the NCBI GenBank sequence database and were found to match with a high degree of specificity to other Arachnids, validating their origin. The gene data were applied to phylogenetic analysis, and the preliminary results clearly highlight potential for future study.

The study recorded almost complete homogeneity of ITS1 sequences both across different populations, and within individuals, in *L.tenuis* sampled from the UK and New Zealand, indicating that the rDNA arrays of these disparate populations have indeed evolved in concert. Whether this is due to the short time in evolutionary terms they have been separated, or due to positive selection for the current spacer sequence in both populations is a matter of conjecture. The *E.ovata* sequences show a slightly higher level of variation between sequences from across Scotland, but show no population differentiation in terms of rDNA variants, with each polymorphism being unique to a single clone (as was the case with all the analysed spider sequences). These data reject the hypothesis presented in the introduction of this chapter which speculated that each *E.ovata* population might homogenise to a different sequence.

Schlotterer and Tautz (1994), in their seminal studies on *Drosophila* ribosomal genes reported that intra-chromosomal homogenisation occurs at a faster rate that inter-chromosomal homogenisation. However, in terms of this study we cannot comment if this is the case, as the origin of the rDNA PCR sequences have not been mapped onto their specific chromosomal ribosomal clusters, and hence we do not know if there is greater homogeneity from sequences from the same cluster, as opposed to clusters from different chromosomes.

There are, however, a number of possible explanations of the pattern of variation recorded. Firstly, the higher number of polymorphisms recorded in the *E.ovata* ITS1 region may be due to the greater effect of genetic drift on small

populations (dictated by their less dispersive nature and habitat requirements). Secondly, it is thought that small populations would also undergo slower homogenisation via gene conversion, thereby allowing any mutations to persist for longer. This possibility is backed up by the limited data generated from *B.gracilis*, which has an intermediate potential for dispersal in comparison with *E.ovata* and *L.tenuis*, and had 2.1 % sequence variation across the three clones from a single individual analysed. The spider results are interesting in the light of the preliminary findings of Navajas *et al.* (unpublished), who found increased variation in the ITS2 region of mite species which had a more restricted dispersal and ecological niche, reflecting the situation with the two spider species studied here. Obviously, more detailed studies are required to elucidate further if these observations have a genuine biological basis. For example, to assess the effects of spatial scale more accurately on genetic differentiation between populations of sessile and vagile spider, a sampling regime with a greater number of sites distributed over a considerably wider geographic area would be undertaken. This would present a greater opportunity for observing population differences between disparate populations, since geographically diverse populations have less opportunity for gene flow. The possibilities for future research are addressed further in Chapter Six.

To recap, whilst proving ineffective as a tool for population differentiation on the geographic scale studied, due to its apparent homogeneity within a species, spider rDNA may prove useful in helping to answer the many unresolved phylogenetic issues in spider biology (Coddington and Levi 1991).

6. GENERAL DISCUSSION

6.1. Applicability of RAPD and rDNA analysis to arachnological studies

The overall aim of this study was to shed light on the largely neglected area of spider population dynamics. By gaining a clearer understanding of what constitutes a spider population (i.e. at what spatial scale populations exist in the agroecosystem), not only will fundamental knowledge be increased, but incorporation of the data into landscape models may lead to better land-management practices which promote these beneficial invertebrates.

However, as the majority of spider species are small and potentially highly dispersive, direct methods of observing population interactions are relatively impracticable. To overcome this problem, a number of DNA based techniques (RAPD analysis, PCR-RFLP, and sequencing) were employed in this study to indirectly infer data on the population structure of two common UK spider species, *L.tenuis* and *E.ovata*. Indeed, whilst there are admittedly problems with the interpretation of indirect estimates of population structure (as discussed by Bossart and Prowell 1998) - e.g. possible natural selection acting on markers, lack of resolution of markers (i.e. retention of shared polymorphisms) and occasional ambiguity between marker systems (e.g. Zink (1986) detected no population structure with the Fox Sparrow populations using allozyme data, but detected a structure using mtDNA (Zink 1994)), indirect methods are often the only practicable manner in which to study highly dispersive invertebrates. Notably, since these two species of spider have widely differing dispersal rates, a contrast of population structures in relation to the dispersal aspect of their life history strategy was possible.

The applicability of the techniques can be assessed in retrospect by referring to the questions originally posed at the outset of the study:

1) How is genetic variation distributed between spider populations, and does this enable estimates of the geographic boundaries of populations, and hence effective dispersal distances, to be made?

2) Are there differences in the genetic diversity and differentiation between *L.tenuis* and *E.ovata* populations which are predictable in terms of their respective dispersal capabilities?

The data generated in this study have successfully taken the first steps towards answering these questions.

As described in Chapter Four, RAPD analysis has been applied by numerous authors to examine population variability because of its ease of application and speed in generating results. It has been equally criticised in some quarters for these very traits, perhaps due in part to the lack of intellectual rigour needed to carry out a RAPD based analysis. However, much of the criticism can be seen as hyperbole. If the limitations of the technique are acknowledged, a considerable level of useful information can be accrued.

In the current study, RAPD-PCR proved a powerful and repeatable method of generating DNA profiles, from adults and spiderlings alike, across a number of spider species. The genetic variation data have enabled an estimate to be placed on the effective genetic population scale of the *E.ovata* samples studied, although broader geographic sampling of *L.tenuis* will be required to elucidate spatial population genetic structure of this dispersive ballooning species. The problems of

repeatability of RAPD results between laboratories cannot be commented on, as inter-laboratory comparisons were not undertaken during the study, but by optimising and maintaining strict protocols and procedures and including the correct controls, repeatability within the laboratory did not present a problem.

RAPD data analysis is an area that is being constantly refined. The methods chosen to analyse the data in this thesis (the generation of Nei and Li similarity matrices, PCO analysis and calculating heterozygosity (gene diversity) and F_{ST} (population subdivision) estimates) are considered appropriate by a large number of authors, although undoubtedly a number of alternative methods could have been undertaken. However, re-assuringly, the results generated in this study reflect the situation which may have been expected intuitively in terms of population differentiation, given the current knowledge of the natural history of *L.tenuis* and *E.ovata* - the ballooning species was recorded as possessing a greater level of heterogeneity within populations and a lower level of structuring between populations than the more sedentary species. This demonstrates that the RAPD-PCR approach successfully addressed the second question posed at the outset of the study, as there are indeed differences in genetic structure which can be related to differences in rates of dispersal.

To summarise this section of the study, as a method of carrying out an initial screen of genetic variability in populations, RAPD analysis, despite its inherent ills, remains a powerful tool - if its assumptions and limitations are borne in mind. Indeed, given the relative geographic proximity of the sample sites, the fact that the analysis of spider populations with only five random primers enabled the populations to be

differentiated, highlights quite how effective and sensitive RAPD analysis can be. And, whilst the RAPD technique is criticised (despite the markers predominantly demonstrating Mendelian inheritance) because allele frequencies and levels of heterozygosity cannot easily be determined, it must be remembered that other approaches are not without their weaknesses also. For instance, allozyme analysis can underestimate genetic diversity because only protein coding loci are assessed, limiting the level of screening (and in addition, not all nucleotide substitutions will result in an amino acid substitution due to degeneracy in the genetic code), whilst PCR based microsatellite analyses can also suffer from problems of reproducibility and be susceptible to changes in PCR conditions e.g. Weising *et al.* 1995.

Ribosomal DNA analysis has also proved effective for many authors in answering questions regarding both the population genetic structure and phylogenetic relationships of many diverse organisms, due to differences in the mutation rates of the elements within each ribosomal array.

In one respect, it could be argued that this section of the present study was unsuccessful, since the primary aim was to examine rDNA in an attempt to detect population specific markers for the spider species studied, which would be utilised in addressing questions regarding population structure. Whilst this did not come to fruition, a number of useful findings did emerge.

The sequence data generated from the ITS1 regions of *L.tenuis* and *E.ovata* (and latterly *B.gracilis*) indicate almost complete homology amongst the ITS1 spacers within the populations sampled, indicating that the phenomenon of concerted evolution is homogenising these spacer repeats. Therefore, conversely, whilst not

immediately useful as population specific markers, the lack of polymorphism in the ITS region within a species suggests that the region may prove a useful phylogenetic marker for future studies within the Araneae. The difference in levels of sequence variation found in the ITS regions of the two species can however, still be hypothesised in terms of gene flow and hence dispersal, which again addresses the second question relating life history strategies to levels of genetic variation.

The production of hypothetical ITS1 secondary structures, which highlighted the conserved nature of the folded ITS1 transcript between spider species, also proved informative. The occurrence of mutations in regions which would least effect the folding of the transcript reinforces that the sequence polymorphisms were genuine and not PCR or cloning artefact. Furthermore, the similarity of the secondary structures between spider species indicates that the ITS1 region may well have a functional role in the processing of the pre-rRNA 45S transcript.

It must be stressed that it would be incorrect to assume that the apparent disparity between the levels of variation detected by the two techniques in any way nullifies the conclusions reached. Ideally, the rDNA results would have reflected those of the RAPD analysis, re-emphasising the relationship between dispersal distances and the lowering of genetic structure. This was obviously not the case, as no genetic structure was found. However, the rDNA is a specific system undergoing a particular set of genetic processes (i.e. molecular drive), which is acting to homogenise the repeated arrays, despite large distances between the sampled populations. It was hypothesised in Chapter One that rDNA homogenisation may

take place at a local scale and hence be of use in differentiating populations. This was found not to be the case.

RAPD data in contrast are generated from anonymous genomic loci, and may have originated from various DNA classes, undergoing different evolutionary processes and cannot therefore be directly compared to the rDNA data. Nonetheless, as discussed in Chapters Four and Five, both the RAPD and rDNA results of *L.tenuis* and *E.ovata* show levels of variation that may be attributable to the different dispersal capabilities and gene flow and can be explained by molecular mechanisms and population level processes.

Perhaps the most obvious, yet simultaneously, the most vexing question regarding DNA variation studies, is what does variation actually mean, in terms of its biological and evolutionary significance to an organism? This is indeed a thorny question which undoubtedly merits a thesis in itself! It has been suggested that organisms with higher levels of genetic variability are more able to cope with changes in environmental conditions (e.g. Martin *et al.* 1997) due to the flexibility that a wider genetic base engenders to a population, and certainly in this study the ubiquitous *L.tenuis* was found to have greater heterogeneity across populations than *E.ovata* as assessed via RAPD analysis. However, this study merely observed and recorded the distribution of polymorphisms within and between populations. Whilst it is impossible to comment on the direct effect these may have on the evolutionary fitness of an organism, detectable polymorphisms do allow researchers to generate measures of similarity, from which a number of hypotheses can be made regarding population structure, breeding systems or phylogenetic relationships.

Notably, molecular data are of no more practical use to ecologists than words on a page make for coherent sentences. Only when DNA data are synergised with data gathered from other branches of ecology and analysed within the framework of life history and natural history studies can they be effectively and practically applied.

Drawing the results from the two sections of the study together, it can be said that a first step has been taken into understanding spider population genetics. It is difficult to make definitive comments regarding any population structure since, depending on the spatial and temporal scale examined, populations typically share attributes of several theoretical types of spatially structured systems. One can step back and point to naiveté in the sampling plan, particularly regarding the search for ITS rDNA polymorphisms, given the relatively restricted geographic range examined. However, given the examples of variation cited in the invertebrate literature, and lack of any current spider data to use as a baseline, the case for exploration was strong. Moreover, the results which were generated during the study have left a number of avenues tantalisingly open for further research, and these will now be discussed.

6.2. Suggestions for future research

This study was, by default, very much preliminary in nature as it was the first to carry out both RAPD analysis and rDNA analysis on spider species and the results and conclusions must be treated as such. Furthermore, the lack of a second year of data to support the conclusions is also a glaring omission which unfortunately could not be rectified in the time available, and aids to reinforce the tentative nature of the

conclusions. Nonetheless, despite these reservations, the study has produced data which now permits future work to be more directed and purposeful.

The RAPD results give a first indication, as may have been intuitively predicted, that spider dispersal by ballooning acts to homogenise the gene pool over relatively short geographic distances, and that the more sedentary species has a more differentiated population structure. The task now would be to enlarge the study not only in terms of geographic range, but also in terms of examining a larger number of spider species with a varied range of life history strategies. Particularly useful would be to conduct a fuller study of *L.tenuis* and *B.gracilis*, to test directly the hypothesis that within the Linyphiidae it is indeed the ballooning frequency that is leading to the homogenisation, and the results were not an artefact of comparing a Linyphiid with a member of the Theridiidae. In actuality, that was the original objective of the project, but when insufficient numbers of *B.gracilis* were found during the first summer sampling period, an alternative sedentary spider had to be chosen and *E.ovata* was a conspicuous and readily available choice. In addition, in any future studies, due to the well documented criticisms of RAPD data, it would also be worthwhile to employ a second DNA fingerprinting method to corroborate results. Given *carte blanche*, microsatellite markers could be generated for the spider species in question as they are considered to be more genetically informative than RAPD data (microsatellites are co-dominant markers which can provide data on zygosity, allowing unbiased population genetic measures to be calculated). If less time was available, anchored-microsatellite primers could be employed, as they have the universality and ease of

application of a random primer (no prior target sequence information is required), mixed with the added degree of specificity which the primer anchoring brings.

The rDNA data were equally revealing - the low levels of variation in the ITS1 regions of spider DNA, garnered both from the RFLP and sequencing studies, indicate quite compelling evidence of the phenomenon of concerted evolution occurring in spiders. The knowledge that these spacer regions are apparently stable within a species, although very different between related genera may enable future studies to employ these regions to answer questions regarding the phylogenetic analysis of closely related spider species. Two exciting examples would be, firstly, to apply ITS1 sequence analysis to species in the two sub-families of the Linyphiidae - the Erigoninae and Linyphiinae, which fall between the two in terms of morphology to help clarify the sysytematics of the family, and secondly, to examine the sister species relationships within the recently hypothesised *E.ovata* group. Moreover, the gene sequence data generated from the spider 28S and 5.8S ribosomal genes can likewise be applied to systematic studies within the Arachnida, which as highlighted in Chapter Five, is an area open to serious investigation.

6.3. Conclusions

(a) The RAPD-PCR technique combines power and simplicity and is an excellent method for producing data on genetic variability in spider populations. Optimisation of the technique is essential for generating reliable and repeatable results.

(b) The data from five primers generated from populations of *L.tenuis* and *E.ovata* were analysed, and reflect what is known of the dispersal ability of those species: The highly dispersive *L.tenuis* is genetically comprised of a relatively undifferentiated population in East Lothian, whilst in comparison, populations of the more sedentary *E.ovata* can be differentiated over a similar geographic range. This can be attributed to differences in levels of gene flow.

(c) The PCR amplification of spider rDNA array with conserved primers was highly successful. Cloning and sequencing of the ITS1 spacer region from different populations of *L.tenuis* and *E.ovata* were carried out to examine variation at the highest resolution..

(d) Analysis, via both PCR-RFLP and sequencing, of the rDNA region of *L.tenuis* and *E.ovata* populations indicates that in both species the ribosomal DNA arrays have been almost completely homogenised to a single repeat sequence, indicative of the phenomenon of concerted evolution. The slightly higher number of unique polymorphisms in the *E.ovata* clones can be hypothesised to have arisen from genetic drift of isolated populations and smaller population size. Whilst not immediately

useful for population level studies at the geographic range examined in this study, the applicability of the technique is not in question, and it is potentially a propitious technique to employ in future phylogenetic studies, or studies using more disparate and remote populations. The hypothetical secondary structures of the spider ITS1 transcripts show considerable homology, indicating its possible functional importance in the processing of the pre-RNA 45S molecule.

(e) In terms of modelling the population dynamics of spider species to aid land-management decisions, this study indicates that *L.tenuis* populations within East Lothian are genetically unstructured, indicative of their ability to disperse and re-colonise disturbed agricultural land. Unfortunately, the sensitivity and scope of the study were not sufficient to predict accurately dispersal distances without further work. It can be said however that dispersal distances of many kilometres cannot be discounted as unusual.

7. BIBLIOGRAPHY

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8. APPENDICES

8.1. Primer Screening

Lepthyphantes tenuis
Bulk screening

AR1-AR20	No amplification 4,5,7,8,and 15
AF4-AF20	No amplification 8 and 9
B01-B20	No amplification 9 and 16

Screened against individuals

Primers highlighting polymorphisms:	AF3,6,15 and AR3,17,18 and 19 B3 and 30
Lack of polymorphism:	AF1,2,12,13,14 and 20 AR1,2,4,5 B4,7,8,11

Enoplognatha ovata
Bulk Screening

OPH1-20	No amplification OPH8 and 10
OPAR19	
OPB03	

Screened against individuals

Interesting primers:	OPH1, 2, 5, 7, 11, 15, 20 OPAR19 OPB03
Insufficient polymorphism:	OPH3, 4, 6, 9, 12, 13, 14, 16, 17, 18, 19

8.2. RAPD population scoring

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Fragment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
AR3 1	0	1	0	1	1	1	1	0	0	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1
2	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	1	0	1	1	0	1	0	1	0	1	1	0	0	0
3	0	0	0	1	0	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	1	0	1	0	1	0	0	1	1	0	1	0	0	1	0	1	0	1	0	0	0	1	0
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7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
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9	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	1	1	1
AR19 10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
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BO3 22	0	1	0	0	1	0	1	0	0	1	1	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	1	0	1	0	0
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B20 29	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0
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44	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0
45	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0
46	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
47	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
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Fragment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
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8.3. Summary of bootstrap analysis of heterozygosity

Summary of bootstrap analysis of gene diversity in *L.tenuis*

Site	Calculated[1] gene diversity (H_w)	Variance of H_w ($\square_{Hw}$)	Mean bootstrap estimate of H_w	Mean bootstrap estimate of $\square_{Hw}$
Spotte	0.313	0.000420	0.3120	0.000393
Phantassie	0.320	0.000500	0.3168	0.000460
Boghall	0.316	0.000460	0.3129	0.000403

[1] Based on 44 RAPD markers and 48 individuals at each site

Summary of bootstrap analysis of gene diversity in *E.ovata*

Site	Calculated[1] gene diversity (H_w)	Variance of H_w ($\square_{Hw}$)	Mean bootstrap estimate of H_w	Mean bootstrap estimate of $\square_{Hw}$
Spotte	0.264	0.0010	0.260	0.0013
Phantassie	0.260	0.0030	0.258	0.0016
Invergowrie	0.203	0.0010	0.200	0.0008
Blackford Hill	0.272	0.0020	0.269	0.0017

[1] Based on 48 RAPD markers and 48 individuals at each site except Phantassie where only 28 indivduals were sampled

H_w= within population gene diversity

$\square_{Hw}$ = Within population variance of gene diversity

1998. The Journal of Arachnology 26:397–400

RESEARCH NOTE

RAPD PROFILING OF SPIDER (ARANEAE) DNA

We present protocols and conditions for specimen storage, DNA extraction and storage, and the subsequent RAPD (Random Amplified Polymorphic DNA) profiling of spiders. Three common UK species, *Lepthyphantes tenuis* (Blackwall 1852), *Enoplognatha ovata* (Clerk 1757) and *Clubiona reclusa* (Cambridge 1863), members of the Linyphiidae, Theridiidae and Clubionidae respectively, were chosen to serve as examples with this highly adaptable technique.

Despite numerous reservations regarding the repeatability, homology, and statistical analysis of the data (see Grossberg et al. 1996 for a comprehensive review), RAPD profiling (Williams 1990) is still the method of choice for many researchers looking to address a wide range of ecological issues in an equally diverse array of organisms. RAPD data have enabled insights into population structure (e.g., Haymer & McInnis 1994; Kambhampati et al. 1992), geographical origins and invasion routes of colonizing species (e.g., Williams et al. 1994), the distinction of new genotypes of parasites (e.g., Majiwa et al. 1993) and conservation genetics (e.g., Rosetto et al. 1995). The RAPD technique can also be a useful initial step in detecting other classes of DNA marker such as microsatellites (Ender et al. 1996).

RAPD profiling is adopted despite the reservations because it possesses many advantages over other molecular marker systems, *viz.,* it is relatively fast and technically undemanding, screens the entire genome for polymorphisms, and can produce a potentially limitless number of markers (simply by screening with more primers). Moreover, due to the amplification process during the PCR thermal cycling, only minute quantities of DNA are required as template, making the analysis of invertebrates unproblematic, e.g., microhymenoptera (Landry et al. 1993).

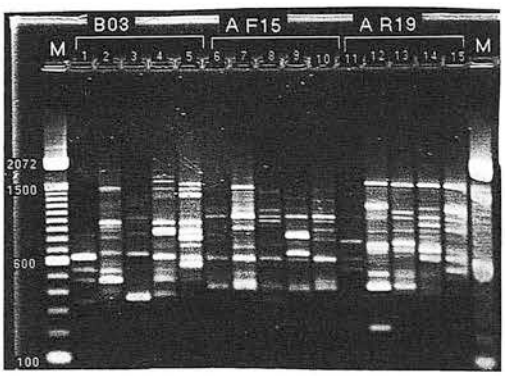
Sample storage prior to DNA extraction was found to be the most crucial stage for this otherwise robust technique, which worked successfully with all the species tested (Fig. 1). Spiders were collected via a D-Vac suction sampler, or by hand, and returned to the lab alive. They were then either stored in ethylene glycol or 70% ethanol at room temperature, or frozen in liquid nitrogen and stored at −80 °C. DNA was extracted after three weeks and examined on a 1% agarose minigel. RAPD reactions were then carried out with DNA stored at 4 °C and −20 °C over a period of one month, to assess the optimal storage for extracted DNA.

Ethylene glycol and 70% ethanol were both found to be poor preservative media for the spider DNA, which had degraded substantially after three weeks storage at room temperature. Storage at −80 °C was found to be the most effective method tested for preserving specimens (at least for one year) prior to DNA extraction if extractions could not be made immediately (Fig. 2). However, it was necessary to identify the spiders prior to storage at −80 °C, as the delicate tissues of the epigyna and palps darkened following freezing, making identification more difficult. Saturated salt solutions have also been used by a number of authors as a means of preserving DNA during field collection of samples, e.g., (Seutin et al. 1991) but these were not investigated in this study.

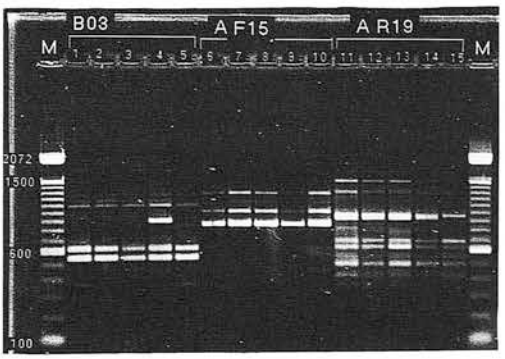
Storage of extracted DNA at −20 °C is recommended if the sample is not to be used directly, as DNA held at 4 °C gave more variable results over time (results not shown). Fresh dilutions of DNA should be prepared from −20 °C stock prior to each RAPD reaction to ensure repeatability of profiles (Fig. 3).

The DNA extraction was carried out as follows. A 1.5 ml Eppendorf tube containing an adult spider was lowered into liquid nitrogen for 10 sec and the spider tipped out onto a Petri dish lid. The abdomen was removed with a sterile scalpel blade, preventing the possible

Lepthyphantes tenuis



Enoplognatha ovata



Clubiona reclusa

Figure 1.—RAPD profiles produced with three primers (chosen at random from those available in the laboratory) from five individuals from each species. Primer sequences are: OPB–O3 (5′–CATCCCCCAG–3′); OPAF–15 (5′–CACGAACCTC–3′) and OPAR–19 (5′–CTGATCGCGG–3′). M = marker (size in base pairs).

amplification of DNA from prey ingested by the spider or of any parasitic burden. The carapace was then returned to the tube and refrozen. The carapace was homogenized with a sterile plastic Eppendorf pestle (a separate pestle was used for each sample to prevent cross contamination), 500μl chilled DNA extraction buffer (200mM Tris-HCl (pH 8.0), 70mM EDTA, 2M NaCl, 20mM sodium metabisulphite) and 90μl 5% sarcosyl solution added (Cheung et al. 1993), then additional grinding carried out to ensure complete destruction of tissue. The addition of Proteinase K and RNAse was not found necessary to extract DNA which amplified to produce clear repeatable profiles. The tubes were then incubated at 65 °C for 1 h with occasional mixing by inversion. Following incubation, the homogenized tissue was spun in a microfuge at 16,000 × *g* for 3 min to pellet gross debris, and the supernatant, containing the DNA, was transferred to a fresh tube. To precipitate the DNA, 90μl of 10M ammonium acetate and 500μl of chilled isopropanol were added to the supernatant, the tube slowly inverted 50 times to mix, and the sample placed at −20 °C for 2 h.

Total precipitated DNA was pelleted at 16,000 × *g* for 10 min, after which the supernatant was poured off and 400μl 70% ethanol added to wash the pellet. Following a further 4 min spin the 70% ethanol was decanted. Finally, the pellet was air dried for 30–45 min then resuspended in 50μl sterile water (Sigma, UK). Resuspension was aided by heating to 60 °C for 1 h. The quantity of the DNA recovered, as observed on a 1% agarose minigel, was comparable with DNA extracted using the more traditional, solvent extraction method, whilst avoiding the unpleasantness of handling phenol and chloroform.

DNA amplification was carried out on a Perkin Elmer TC-1 thermal cycler, using a step cycle, programmed for 35 cycles of 1 min at 95 °C for DNA denaturation, 1 min at 36 °C for primer annealing, and 2 min at 72 °C for primer extension. This was preceded by an initial denaturation step of 2 min at 95 °C. The cycling was followed by a final primer extension step at 72 °C for 8 min. Following optimization of DNA and magnesium concentrations, in a 50μl reaction volume the following components were employed: 1X Perkin Elmer

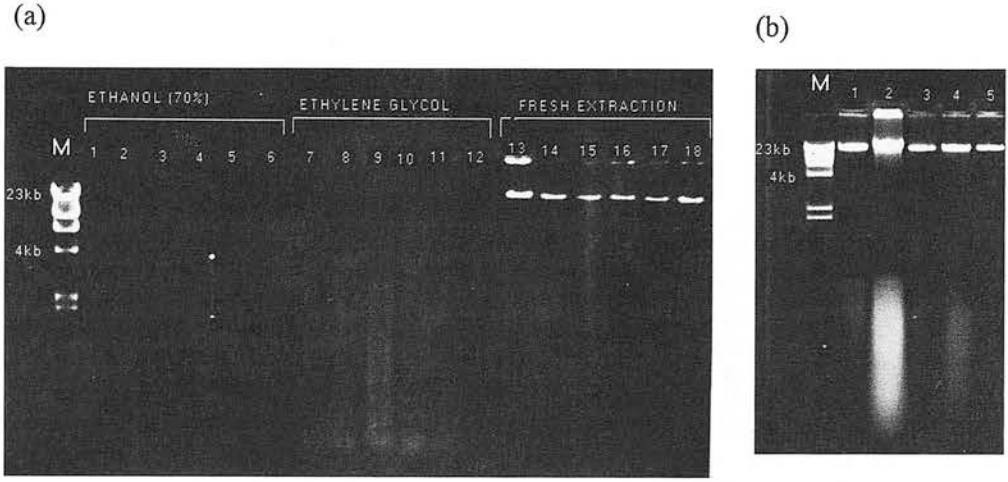


Figure 2.—Effect of specimen preservation on DNA. (a) DNA extractions from 18 *Lepthyphantes tenuis* stored for three weeks at room temperature in 70% ethanol (lanes 1–6), ethylene glycol (lanes 7–12, or recovered from fresh specimens (lanes 13–18). (b) Extraction from 5 *L.tenuis* stored at −80 °C for 12 months. M = marker (size in base pairs).

buffer, 3mM $MgCl_2$, 200μM each of dATP, dTTP, dCTP and dGTP, 0.5 units of Stoffel *Taq* and 0.2μM primer (10-base primers, Operon Technologies Inc., Alameda, California, USA). DNA template was present at a concentration of approximately 40ng per reaction, calculated by comparing by eye the intensity of ethidium bromide stained genomic extracts with dilutions of a DNA marker (λ/HindIII digest) whilst under UV illumination (Sambrook et al. 1989). This allowed dilutions of DNA to be made which were in a good approximation to each other. Finally, prior to PCR, the reaction mix was overlaid with approximately 25μl of mineral oil to prevent evaporation of the sample during cycling.

Amplified RAPD products were visualized on a 1.5% TAE agarose gel following electrophoresis at 80 volts for 2 h. The gel was stained with ethidium bromide (0.5μg/ml) for 20–30 min, rinsed briefly, then examined on a UV illuminator. The results were captured using the IS500 digital image analysis system (Flowgen, UK).

Sample storage in ethanol for future DNA extraction is something of a contentious issue, with reports ranging from vertebrate tissues stored for six years producing good yields of high molecular weight DNA (Smith et al. 1987), to Coleopteran DNA which maintained its integrity for only six weeks in 95% ethanol (Reiss et al. 1995). Laulier et al. (1995) state

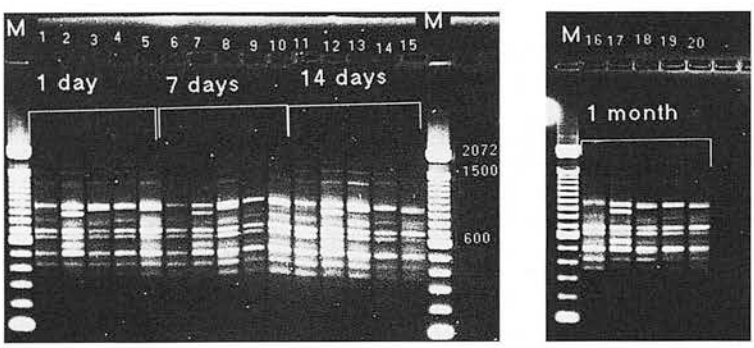


Figure 3.—Reproducibility of RAPD markers over time. Profiles from stock DNA extractions stored at −20 °C with primer, OPAR–19. Five *Enoplognatha ovata* after 1 day (lanes 1–5), 7 days (lanes 5–10), 14 days (lanes 11–15) and one month (lanes 16–20). M = marker (size in base pairs).

that DNA can be recovered from ethanol and methanol preserved samples, but the degree of degradation appears to be species specific, and the yield is generally poor. It can be speculated that any species specificity of degradation may be due to the physical properties of the cuticle of the organism. Ito (1992) reported that unknown contaminants in 100% ethanol can cause degradation of DNA, leading to the simple classification of ethanols as "good" and "bad". Our findings support the difficulty of finding a "good" ethanol and it may be prudent not to take the risk if possible.

In summary, this preliminary study has shown that following optimization, the RAPD technique produces clear and repeatable results and is readily applicable to arachnological studies. Molecular data from such studies should allow new insights into a number of ecological issues if applied appropriately.

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